

Microbial Production and Characterization of 1,3-PDO
by a Novel *Lactobacillus panis* Strain

A Thesis Submitted to the College of Graduate Studies and Research
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in the Department of
Food and Bioproduct Sciences
University of Saskatchewan
Saskatoon

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2012

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ABSTRACT

Interest in the aliphatic carbon compound 1,3-propanediol (1,3-PDO) has risen over the past 15 years. In part, this interest is due to the ability of 1,3-PDO to generate a variety of industrially relevant products such as the biodegradable polymer, polytrimethylene terephthalate. Our research group previously reported the identification of a novel *Lactobacillus panis* PM1 isolate capable of converting glycerol to 1,3-PDO. In this body of work, the effects of various process parameters and the ability of the novel *L. panis* isolate to produce 1,3-PDO in static and fed-batch cultures were examined. Data collected indicated that the concentrations of glycerol, and glucose, and pH, play a vital role in the optimized production of 1,3-PDO. Optimal conditions for the production of 1,3-PDO were determined to include: i) carbon-limited culture, defined as below 50 mM glucose and ii) growth at 37°C without agitation in the presence of glycerol (150 – 250 mM) at an elevated pH of 9 – 10. Factors such as inoculum size and temperature (OD_{600} in the range of 0.5 – 2 and a temperature range from 15° - 37°C) in a two-step fermentation showed insignificant variance in the production of 1,3-PDO. Initial fed-batch trials reflected the importance of pH on culture viability. A pH of 8 was determined to be necessary within culture parameters for the fed-batch production of 1,3-PDO. Further, the molar concentrations of 1,3-PDO produced were found to vary only slightly between fed batch culture and a static culture. The variance of 1,3-PDO production between the static and fed-batch trials was found to be 9.1 ± 4.9 mM for an average culture producing 85.3 ± 12.0 mM of 1,3-PDO. However, the mol concentrations of 1,3-PDO produced were found to be significantly higher with 22.3 ± 1.6 versus 5.3 ± 0.7 mmol 1,3-PDO produced for the fed batch versus the static cultures, respectively. The duration of 1,3-PDO production was found to be extended in the fed-batch model of production with increased levels of 1,3-PDO being produced over 120 hours. The cloning and characterization of the recombinant 1,3-PDO NAD^+ -dependent oxidoreductase also were explored to gain further insight into the native production of 1,3-PDO. Initial kinetic studies determined a K_m value of 1.28 ± 0.57 mM for NAD^+ versus 23.8 ± 1.1 mM

for 1,3-PDO. The K_m values demonstrated that the availability of NAD^+/NADH may be a determining factor in 1,3-PDO concentration. These findings support the literature and the conclusion that the bottleneck in 1,3-PDO production lies in maintaining an available pool of NAD^+/NADH while mitigating negative effects associated with the accumulation of toxic byproducts.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to Dr. Takuji Tanaka for his guidance, support, and supervision throughout the course of my project. The insight and guidance I have received from my committee members Dr. Darren Korber, Dr. Martin Reaney, Dr. Vladimir Vujanovic, my external examiner Dr. Steven Siciliano, and Dr. Bob Tyler have been invaluable in the completion of my project.

Working in the lab has been a tremendous experience and would not have been so if it wasn't for the friendship and assistance I received from various colleagues. To my lab mates and fellow graduate students thank you for your kindness, support, and generosity. I wish you all the best and hope to stay in touch with you all in the future!

Finally I would like to thank my Mom, Step-dad, brothers, girlfriend, and dedicate this work the memory of my late father. Mom, you always thought I'd end up taking things apart and putting them back together. Kenny you have always there for me and your support and encouragement has always meant the world to me. Kristina, none of this would have been possible without your love and encouragement. I'll never be able to thank you all sufficiently for everything, or show you how much it has meant to me, but thank you from the bottom of my heart.

TABLE OF CONTENTS

PERMISSION TO USE	i
ABSTRACT.....	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
LIST OF EQUATIONS.....	xiii
 1. INTRODUCTION	 1
2. LITERATURE SURVEY	3
2.1 The Economics and Geopolitical Motivation Behind Energy and Renewable Resources Management – A Focus on Biofuels	 3
2.2 Bioethanol and Biodiesel – A Review	5
2.2.1 Bioethanol	6
2.2.2 Biodiesel	9
2.3 Biorefineries and Glycerol as a Path to Success for the Biofuel Industry	11
2.3.1 Biorefineries: A definition	13
2.3.2 Using Glycerol to Produce High Value Added By-products	16
2.4 Microbial production of 1,3-Propanediol	19

2.5 Objectives and Project Overview	24
3. STUDY 1 – OPTIMIZATION AND CHARACTERIZATION OF 1,3-PROPANEDIOL PRODUCTION FROM A NOVEL <i>LACTOBACILLUS PANIS</i> PM1 ISOLATE	25
3.1 Abstract	25
3.2 Introduction.....	26
3.3 Materials and Methods.....	29
3.3.1 Chemicals and Instruments	29
3.3.2 Determination of <i>L. panis</i> PM1 cellular growth, 1,3-PDO, and major metabolite concentrations	29
3.3.3 Metabolite concentrations during initial 1,3-PDO production	30
3.3.4 Examination of carbon sources capable of producing 1,3-PDO.....	31
3.3.5 Effects of culture atmosphere conditions on 1,3-PDO production	31
3.3.6 Effects of initial glycerol concentration on 1,3-PDO production	31
3.3.7 Effects of initial pH on 1,3-PDO production	32
3.3.8 Effects of temperature on 1,3-PDO production	32
3.3.9 Effects of initial cell density on 1,3-PDO production	32
3.4 Results	33
3.4.1 Metabolite concentrations during initial 1,3-PDO production	33
3.4.2 Production of 1,3-propanediol by <i>L. panis</i> PM1 utilizing various carbon sources ...	34
3.4.3 Effects of culture atmosphere conditions on 1,3-PDO production	35
3.4.4 Effects of initial glycerol concentration on 1,3-PDO production	40
3.4.5 Effects of initial pH on 1,3-PDO production	41
3.4.6 Effects of temperature on 1,3-PDO production	43
3.4.7 Effects of initial cell density on 1,3-PDO production	44
3.5 Discussion	45
3.6 Conclusion	51
3.7 Connection to Next Study.....	52
4. STUDY 2 – FED-BATCH PRODUCTION OF 1,3-PDO FROM <i>L. PANIS</i>	53
4.1 Abstract	53

4.2 Introduction.....	54
4.3 Materials and Methods.....	56
4.3.1 Analytical Determination and Quantification of Cellular Growth, 1,3-PDO, and Major Metabolite Concentrations.....	56
4.3.2 Fed-Batch production of 1,3-propanediol	56
4.4 Results	58
4.4.1 Fed-Batch production of 1,3-propanediol	58
4.5 Discussion	62
4.5 Conclusion	64
4.6 Connection to Next Study	65
5. STUDY 3 – CLONING AND CHARACTERIZATION OF NAD ⁺ DEPENDENT OXIDOREDUCTASE FROM <i>LACTOBACILLUS REUTERI</i> DSM 20016.....	66
5.1 Abstract	66
5.2 Introduction.....	67
5.3 Materials and Methods.....	68
5.3.1 Cloning of 1,3-propanediol NAD ⁺ dependent oxidoreductase	68
5.3.2 Expression of recombinant 1,3-PDO oxidoreductase	71
5.3.3 Determination of recombinant oxidoreductase kinetics	71
5.4 Results	73
5.4.1 Expression of recombinant 1,3-PDO oxidoreductase	74
5.4.2 Determination of recombinant oxidoreductase kinetics	76
5.5 Discussion	78
5.6 Conclusion	80
6. GENERAL DISCUSSION.....	81
7. GENERAL CONCLUSIONS.....	83
8. REFERENCES	86
9. APPENDIX A – CLONING OF THE A AND B SUBUNITS OF GLYCEROL DEHYDRATASE FROM <i>L. REUTERI</i> DSM20016	94
10. APPENDIX B – NMR SPECRTRA OF <i>L. PANIS</i>	100

LIST OF TABLES

Table 2.1 Sugar-based chemical byproducts of interest for biorefineries.....	15
Table 3.1 Re-suspension of <i>L. panis</i> PM1 in mMRS (100 mM glucose and 200 mM glycerol) and the associated production of 1,3-PDO, ethanol, OD ₆₀₀ , and glucose consumption (N = 2)	36
Table 3.2 Production of 1,3-PDO over 24 hours in a culture of <i>L. panis</i> grown in MRS and resuspended in mMRS containing 100 mM glucose and 200 mM glycerol.....	37
Table 3.3 Production of ethanol over 24 hours in a culture of <i>L. panis</i> grown in MRS and resuspended in mMRS containing 100 mM Glucose and 200 mM glycerol	37
Table 3.4 Effects of aeration on the production of 1,3-PDO in a <i>L. panis</i> PM1 culture re-suspended in mMRS.....	39
Table 4.1 Production of 1,3-PDO in initial fed-batch pH 10 and 9 Trials	58
Table 5.1 Primers utilized for cloning of the NAD ⁺ dependent oxidoreductase.....	70
Table 5.2 Kinetics of recombinant NAD ⁺ oxidoreductase	77

LIST OF FIGURES

Figure 2.1 Overview of Conversion routes to Biofuels.....	6
Figure 2.2 The development of the share of biotechnological processes in the sales volume of chemical by-products groups in 2004 and 2015	13
Figure 2.3 Main Pathways for the Utilization of Glycerol	17
Figure 2.4 Derivatives of glycerol – circled chemicals in commercial use at commodity-scale levels of production.....	17
Figure 2.5 Mechanism of glycerol dehydratase reaction.....	22
Figure 3.1 The change in metabolite concentration (1,3-PDO, glucose, glycerol, and ethanol) and growth for a culture of <i>L. panis</i> re-suspended in mMRS over 24 hours at 37°C without agitation	34
Figure 3.2 The effect of carbon source on 1,3-PDO production	38
Figure 3.3 The effect of carbon source on the growth of <i>L. panis</i> PM1.....	38
Figure 3.4 The effect of glycerol concentration on the production of 1,3-PDO..	41

Figure 3.5 The effect of pH on 1,3-PDO production in <i>L. panis</i> PM1.	42
Figure 3.6 The effect of pH on the growth of <i>L. panis</i> PM1 re-suspended in mMRS media.	43
Figure 3.7 Proposed pathway for the conversion of glycerol to 1,3-PDo and the role of glycerol in aerobic metabolism.....	50
Figure 4.1 Hydroformylation of ethylene oxide, (A) Shell method. (B) Degussa conversion of acrolein to 1,3-PDO	54
Figure 4.2 Effect of pH and glucose concentration on the fed-batch production of 1,3-PDO by <i>L. panis</i> PM1.....	59
Figure 4.3 Effect of pH and glucose concentration on the fed-batch production of 1,3-PDO by <i>L. panis</i> PM1.....	60
Figure 4.4 Effect of pH and glucose concentration on the growth of a fed-batch culture of <i>L. panis</i> PM1.....	61
Figure 5.1 Restriction digestion of pET-32(b)+ candidate NAD ⁺ dependent oxidoreductase clones	73
Figure 5.2 A 10% SDS-PAGE gel of candidate recombinant NAD ⁺ dependent oxidoreductase clones grown and expressed at 37°C..	74
Figure 5.3 10% SDS-PAGE gel of candidate recombinant NAD ⁺ dependent oxidoreductase clones grown and expressed at 20°C.....	75
Figure 5.4 Activity of recombinant 1,3-PDO NAD ⁺ dependent oxidoreductase clones	76

Figure A.1 Expression of recombinant glycerol dehydratase in pKK223-3	94
Figure A.2 Determination and solubilization of inclusion bodies expressed by recombinant glycerol dehydratase in pKK223-3.....	95
Figure A.3 Determination and solubilization of inclusion bodies expressed by recombinant glycerol dehydratase in pKK223-3.....	96
Figure A.4 Mass spectrometry result of the dominant protein band in the 5M urea soluble fraction	97
Figure A.5 Mass spectrometry result of the dominant protein band in the 1M urea pellet fraction	97
Figure A.6 Expression of alpha (GD) and beta (GDA) subunits of glycerol dehydratase in the pHIL-S1 clone	98
Figure A.7 pET-32b(+) expression of alpha subunit (GD) and glycerol	99
Figure B.1 NMR Spectra of <i>L. panis</i> grown in MRS and resuspended in mMRS at 0 and 24 hours	100
Figure B.2 NMR Spectra of <i>L. panis</i> grown in MRS and resuspended in mMRS at 1 and 2 hours	101
Figure B.3 NMR Spectra of <i>L. panis</i> grown in MRS and resuspended in mMRS at 1 and 4 hours	102

Figure B.4 NMR Spectra of <i>L. panis</i> grown in MRS and resuspended in mMRS at 1 and 2 hours ...	
.....	103

Figure B.5 NMR Spectra of <i>L. panis</i> grown in MRS and resuspended in mMRS at 1 and 4 hours ...	
.....	104

LIST OF EQUATIONS

Equation 2.1 Summarized conversion of a sugar or carbohydrate to ethanol7

Equation 2.2 Conversion of sucrose to glucose and fructose8

Equation 2.3 Conversion of glucose to ethanol and carbon dioxide8

Equation 5.1 Re-arrangement of Beers law to determine the activity of recombinant 1,3-PDO
NAD⁺-dependent oxidoreductase77

1. INTRODUCTION

Rapid advancements in technology in a variety of commercial sectors, up until the first half of the 20th century, proceeded regardless of their associated environmental impact (Clark 1999). As a result, many of the technologies used to generate the literal and figurative synthetic fabrics of modern society such as petrochemicals, pharmaceuticals, automobiles, laptops, tablets, and countless other disposable goods, produce environmentally harmful pollutants and/or are dependent upon a finite resource(s) (Gungor and Gupta 1999). Considering how these products permeate modern society, alternative resources and methods for their production must be discovered and developed before environmental concerns and product costs begin to drastically and negatively affect the world in which we live.

In an effort to decrease their environment impact many industries are focusing on environmentally friendly or green models of production (Gungor and Gupta 1999). The goal of a green model of production is to design or generate chemical products and processes that eliminate the use or generation of hazardous substances (Anastas and Kirchhoff 2002). As such, research into areas such as polymers, catalysis, biobased/renewables, synthetics, analytical methods, and safer chemicals have blossomed over the years (Anastas and Kirchhoff 2002). The rise in research related to green chemistry is not only a result of the need to develop alternative environmentally friendly processes but the economical and sociological cost associated with not doing so (Clark 1999; Gungor and Gupta 1999).

A biorefinery is, in essence, a means of generating a primary and secondary product of interest from a renewable biomass conversion (Demirbas 2009). Biorefineries have been suggested as a means of obtaining an economically viable, environmentally friendly, and renewable series of products. Bioethanol and biodiesel rank amongst the highest primary products of interest for biorefineries given the increasing demand for biofuels and their associated economic dividends (Ragauskas et al. 2006; Taylor 2008; Demirbas 2009). Although a

number of secondary products may be produced alongside bioethanol and biodiesel, 1,3-propanediol (1,3-PDO) is consistently suggested as a key chemical given the production target of 1.5 billion lb/yr in 2004 (Werpy 2004). Interest in the production of 1,3-PDO stems from its ability to act as a platform chemical in the production of plastics and nylon (Werpy 2004). It has been determined that various microbial species are capable of fermenting the glycerol produced as a waste product during the production of biofuels to 1,3-PDO (Pagliaro et al. 2007). By providing an alternative means of producing plastics and nylons from a renewable resource, with minimal chemical synthesis required, industry is taking a step forward to alleviating industrial demands in an ecological safe manner.

Unfortunately, the microbial production of 1,3-PDO from glycerol is still inefficient and, as of yet, is unrealized at an industrial level. However, a number of studies have been conducted examining the capability of various microbes to convert glycerol to 1,3-PDO at levels sufficient for industrial utilization (Menzel et al. 1997; Willke and Vorlop 2008; Rathnasingh et al. 2009; Xue et al. 2010). Two of the primary microbial candidates for the production of 1,3-PDO from waste glycerol are *Citrobacter freundii* and *Klebsiella pneumoniae* due to their substrate tolerance, yield, and productivity (Saxena 2009). However, *C. freundii* and *K. pneumoniae* require specific safety precautions given their status as opportunistic pathogens. The work contained within aims at examining and optimizing the production of 1,3-PDO from glycerol by a novel *L. panis* isolate, which is a lactic acid bacterium (LAB) and hence has a GRAS status. Given that the yield of 1,3-PDO produced is dependent on the process conditions various factors thought to affect the production of 1,3-PDO will be examined (Biebl 1999). By gaining a further understanding of the various factors that affect the microbial production of 1,3-PDO it is believed that the novel isolate, *L. panis* PM1, or the data obtained, will aid in the production of environmentally friendly products.

2. LITERATURE SURVEY

2.1 The economic and geopolitical motivation behind energy and renewable resources management – A focus on biofuels

Global energy consumption is expected to be 605.1 exajoules per year by 2015 (Us-Eia 2011). An assessment of global energy consumption performed by the United States Energy Information Administration (US-EIA) expects this number to rise at an annual rate of 1.5% to 760.7 exajoules per year by 2030 (Us-Eia 2011). Liquid petroleum and non-petroleum based fuels currently encompass, and are expected to continue to encompass, the predominant supply of the world's energy to 2030. In the liquid fuels category, traditional and non-traditional non-renewable based fuels, such as bioethanol and biodiesel, are expected to remain the largest contributors to this group.

Annual consumption of traditional and non-traditional non-renewable based fuels is expected to grow from 30.81 billion barrels per year in 2010 to 36.76 billion barrels per year in 2030, or 188.5 exajoules per year to 224.9 exajoules per year, respectively (Us-Eia 2009). With global energy demands increasing 33% over the next 20 years current energy production in liquid and non-liquid fuels must increase substantially to meet the projected demand. Notwithstanding the difficulties of increasing the levels of production for a declining finite resource the current energy system is unsustainable because of equity issues as well as environmental, economic, and geopolitical concerns that have far reaching implications (Edwards 2002; Demirbas 2009; Nel and Van Zyl 2010).

Of the major anxieties limiting the current energy system, economic and geopolitical concerns appear to be the driving forces in the search for a viable and renewable alternative liquid fuel (Agarwal 2007; Yazdani and Gonzalez 2007; Demirbas 2009). Economic concerns over the current global energy system stem from the fluctuating prices of petroleum fuels in current and future markets along with anxiety over the decreasing availability of non-renewable liquid

and non-liquid fuels alike. As a result economic growth models explicitly based on energy consumption have been developed and refined (Nel and Van Zyl 2010). Utilizing an explicit energy-based economic growth formulation, one can forecast potential economic scenarios till 2050. Current empirical data predicts the peak and decline of primary energy resources between 2010 and 2030 (Edwards 2002; Nel and Van Zyl 2010). Using the predicted peak and decline of primary energy resources in the explicit energy-based economic growth formulation, a deepening recession is forecast until 2050 (Nel and Van Zyl 2010). Moreover, it is expected that difficulties in locating and extracting energy resources will lead to a decrease in the profit-ratio for non-renewable fuel sources. Given the forecast from the economic models and a decreased profit ratio expected for non-renewable energy resources it should come as no surprise that economic concerns are strong motivating factors in the search for a viable alternative renewable energy source. Short term effects due to increased oil prices are just as worrisome as a deepening recession as it has been shown that up to 25% of the employment variability seen in the manufacturing industries comes as a result of rapid changes in oil prices (Davis and Haltiwanger 2001). In addition, it has been found that short term job loss is much more sensitive to rapid changes in oil prices whereas job creation shows no such equilibrating rise during decreases in oil prices. In addition to the fluctuating and increasing prices of liquid fuels the anticipated loss of oil and natural gas reserves in 41 and 63 years, respectively, add to the economic concerns of the worlds' energy system (Agarwal 2007). All of these factors significantly contribute to the economic pressures associated with the search for and development of an alternative renewable energy source. As a means of stemming the negative effects of the current global energy system governments have been, and have begun, to enact more pro-active policies in relation to their foreign dependence on fuels and their development of renewable fuel stocks.

The 1973 oil crisis exposed the worlds' major powers dependence on imported oil. The US, under the Nixon administration, implemented "Project Independence" calling for energy self-sufficiency by 1980. Brazil responded to the oil embargo in a similar manner by beginning a switch to bioethanol as an alternative to imported oil. Currently, the US is more dependent on foreign oil than it ever has been, with US weekly imports of crude oil and petroleum having

increased from ~6,000,000 – 9,000,000 barrels of oil per day in 1991 to ~11,000,000 - 14,000,000 barrels of oil per day in 2008 (Us-Eia 2009). The member states of the European Union (EU) have not fared better as their total oil imports have continued to increase since 2001 with estimated imports reaching ~18,000,000 barrels of oil per day in 2008 (Us-Eia 2009). Conversely, the Brazilian governments initiative to switch to bioethanol has cost the Brazilian government 11 billion dollars in bioethanol investments as of 2005, but has saved an estimated 27 billion dollars in oil imports (Pessoa et al. 2005). In conjunction with rising dependence on foreign oil and rapid changes in oil prices major oil importers such as the EU and the US are now, more than ever, pushing for the development of alternative renewable fuels. Both the US and the EU have set policies as of 2009 that call for the advancement of biofuel research and commercialization (Demirbas 2008; Secretary 2009). Specifically, the EU has called for the use of 23% of total transportation fuel to be comprised of alternative fuels by 2020 (Demirbas 2008). Whereas, the US, under the Recovery Funds act, has committed 786.5 million dollars towards biofuels research and commercialization (Secretary 2009). Canada has committed 1 billion dollars total to next generation biofuels and clean energy fuels under the Next Gen Biofuels Fund and the SD Tech Fund (Sdt-Canada 2011).

The commitment to alternative fuels shown in recent years by the worlds' major oil importers demonstrates the increasing importance of biofuels and alternative renewable fuels as a whole (Sparks and Ortmann 2011). Global fuel consumption of biofuels is expected to rise 8.6% per annum over the next 20 years to 5.9 million barrels per day by 2030 (Us-Eia 2009). If expectations are met biofuels would be second only, in terms of world liquid fuel production, to conventional liquid fuels by 2030. The amalgamation of various economic, geopolitical and energy demands place biofuels and alternative fuels as a top priority in the coming century.

2.2 Bioethanol and biodiesel - A review

Biofuels have been defined as, liquid or gaseous fuels for the transport sector that are predominantly produced from biomass (Demirbas 2008). Depending on the source of the biomass and method of conversion, biofuels can be separated into particular categories such as: biodiesel, pyrolysis liquids, green diesel, green gasoline, butanol, methanol, syngas liquids,

biohydrogen, algae diesel, algae jet fuel, hydrocarbons, and bioethanol further subdivided into sugar ethanol, cellulosic ethanol, and grain ethanol (Demirbas 2009). Of these biofuels biodiesel and bioethanol have emerged as the most promising biofuel technologies (Agarwal 2007; Jegannathan et al. 2009). With bioethanol and biodiesel having emerged as the predominant renewable biofuel technologies the political and economic impetus has been on their use and commercialization. With the impetus for a renewable biofuel placed on bioethanol and biodiesel their associated methods of biomass to biofuel conversion have dominated renewable fuel research. As can be seen in Figure 2.1, a variety of methods and modes of production for the varying biofuels do exist. Due to the preeminence of bioethanol and biodiesel and their associated hydrolysis and fermentative methods of conversion focus will be placed on these methods in particular.

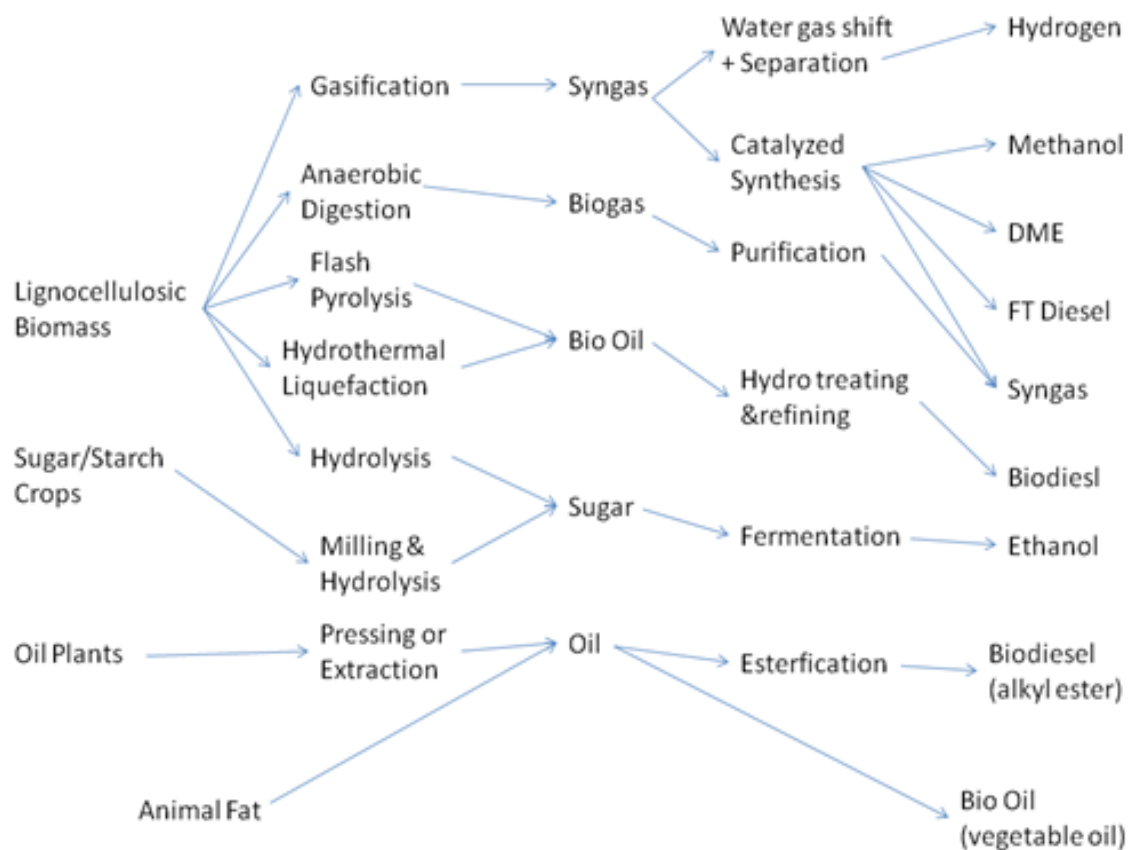


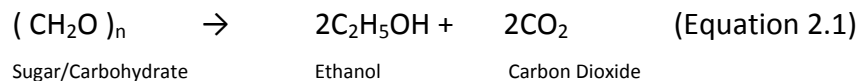
Figure 2.1 Overview of Conversion routes to Biofuels

2.2.1 Bioethanol

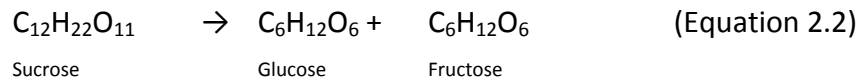
Alcohols derived from renewable biomass (ethanol, methanol, propanol, and butanol) may be used as alternatives to traditional fuel for internal combustion engines (Demirbas 2008). Of these alcohols only ethanol and methanol have been found to be suitable alternatives for current internal combustion engines due to technical and economical reasons (Demirbas 2008). Of the two alcohols, methanol is generally seen as an inferior alternative fuel due to issues of toxicity, cleanliness, and its corrosive nature in comparison with ethanol (Agarwal 2007). Bioethanol is seen as superior to methanol and gasoline due to its higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization (Balat et al. 2008). However, bioethanol is not without problems as low energy density, low vapor pressure, high associated costs and toxicity to ecosystems, due in part to its miscibility with water, are all major disadvantages of bioethanol (Balat et al. 2008). Fortunately, many of these issues, such as low energy density and low vapor pressure, are offset by the advantages of bioethanol such as its high octane number or through technological innovations such as improvements to ignition and glow-plug/spark-plugs, respectively (Balat et al. 2008). Eliminating these potential issues only helps to support bioethanol as a prominent renewable fuel source. The production of bioethanol from a biomass to an alternative fuel consists of four distinct areas: (i) feedstock choice and treatment, (ii) hydrolysis of sugars to glucose *via* enzymatic or chemical catalysis, (iii) fermentation of sugars to ethanol, and (iv) distillation of ethanol to produce absolute ethanol (Jegannathan et al. 2009).

2.2.1.3 Ethanol fermentation

Ethanol fermentation relies on the basic principal that a sugar, in this case glucose, sucrose a dimer composed of glucose and fructose and fructose itself, through a series of biocatalysts can produce ethanol and carbon dioxide. The general conversion of a sugar or carbohydrate to ethanol can be summarized in Equation 2.1.



The primary biological catalyst employed to convert the carbohydrates into ethanol is the budding yeast *Saccharomyces cerevisiae*. The chemical conversion of sucrose to ethanol occurs through a dual-enzymatic system. The enzyme invertase catalyzes the hydrolysis of sucrose into glucose and fructose monomers as seen in Equation 2.2. The enzyme complex zymase then catalyzes the conversion of glucose and fructose to bioethanol as seen in Equation 2.3.



The ability of *S. cerevisiae* to convert sugars to ethanol is exploited in one of two sequential processes. A separate hydrolysis and fermentation (SHF) configuration employs the hydrolysis and fermentation of the sugars in different reactors. Conversely a simultaneous saccharification and fermentation (SSF) configuration implies the hydrolysis and fermentative steps are carried out in the same reactor. A SHF configuration allows for the optimal configuration of both the hydrolysis and fermentative steps due to its sequential nature (Sanchez and Cardona 2008). A SSF configuration has the advantages of higher ethanol yields and lower energy demands (Sanchez and Cardona 2008). The advantages in terms of ethanol yield for the SSF system is due to the simultaneous conversion of hydrolyzates into ethanol and carbon-dioxide. The immediate conversion of the hydrolyzate to ethanol reduces the accumulation of potential inhibitors (Sanchez and Cardona 2008).

Following fermentation the fermented solution, often termed beer, contains a certain percentage of ethanol and water. In most cases the beer contains on average 5 – 12 wt% ethanol (Huang et al. 2008). The distillation of the beer to produce absolute ethanol is hindered by the presence of water as it forms an azeotropic mixture. In order to properly purify the ethanol from solution two distillation steps are required (Huang et al. 2008). The first distillation

is termed an ordinary distillation. Ordinary distillation takes advantage of the low boiling point of the ethanol water azeotrope under slight pressure. By heating the fermented solution the ethanol water azeotrope forms a vapor which is collected and then condensed. This condensed vapor is often referred to as low proof alcohol and can contain up to 92.4 wt% ethanol (Huang et al. 2008). The low proof alcohol is then passed through the secondary distillation step to further purify the solution. The distillation of the low proof alcohol can be processed in a number of ways. Two of the most common methods of distillation are *via* a rectifier column or through azeotropic distillation. A rectifier column is similar to ordinary distillation in its heating of the ethanol water mixture to produce a vapor. Typical rectifier systems can reach levels of 95 wt% purity. However a rectifier system requires a specific condenser and a further dehydration process to achieve 99.9 wt% purity. Azeotropic distillation involves the addition of a further chemical, termed an entrainer, to modify the chemical properties of the water ethanol azeotrope (Huang et al. 2008). By modifying the chemical properties of the azeotrope the relative volatilities of the system are altered along with their separation factors (Huang et al. 2008). Once the entrainer is added to the system the tertiary azeotrope is filtered through a dehydration column to remove the water. With the water removed the ethanol-entrainer solution is then run through an entrainer recovery column to remove the entrainer leaving the final ethanol product pure.

2.2.2 Biodiesel

Biodiesel is defined as the esterification and transesterification of free fatty acids and triglycerides (Mcneff et al. 2008) for use as a diesel fuel alternative (Demirbas 2005). Implied in this statement is the use of free fatty acids and triglycerides as the biomass to be converted to a fuel source. Further implied, but not explicitly stated, is that the removal of the glycerol backbone and slight chemical modification of triglycerides and free fatty acids, respectively, generates a liquid fuel capable of powering a diesel engine. As in the production of bioethanol a variety of crops exists that are capable of providing the triglycerides needed for biodiesel production. Additionally, biodiesel is capable of utilizing animal fats, acidulated soapstocks and waste vegetable oils as potential biomass sources (Demirbas 2005; Mcneff et al. 2008). The

mode of production and biomass of biodiesel confer a number of advantages over traditional diesel fuel and other alternative fuel sources. Most notably biodiesel has the distinct advantage over fossil fuels of; ready availability, renewability, high heat content, low sulfur content, lower aromatic content and biodegradability (Mcneff et al. 2008). Offsetting these advantages are problems of high viscosity, lower volatility, costly feedstocks and the reactivity of unsaturated hydrocarbon chains. However, the preeminent advantage associated with biodiesel is its energy return of 93% which is far superior to the 25% estimated for bioethanol (Mcneff et al. 2008). Biodiesel shares further similarities with bioethanol in its mode of production as: (i) choice of feedstock, (ii) transesterification and esterification of biomass, and (iii) purification are all distinct and vital areas for the production of biodiesel.

2.2.2.3 Biodiesel purification

Purification of biodiesel is a necessary step in the manufacturing process as laid out by the European Standard for biodiesel (Berrios and Skelton 2008). Purification of biodiesel refers to the separation of the alkyl esters from impurities such as free fatty acids, water, methanol, glycerol and metals (Berrios and Skelton 2008). Impurities such as the above can greatly affect both engine performance and generate issues of biodiesel storage and transport. Due to the nature of the chemicals and components used and generated in the esterification and transesterification reaction purification is a relatively easy two step procedure. The first step in purifying biodiesel is the removal of the alcohol and glycerol by-product. A distillation step can be employed before separating the glycerol phase from the impure alkyl ester solution. Distillation refers to the separation of the alcohol remaining in solution by a heating step. The heating step removes and recycles any remaining alcohol back into the process by heating the impure solution. Due to its low boiling point the alcohol in solution will evaporate out of solution allowing for the vapour to be condensed and collected. Removal of the glycerol by-product is made possible by differences in density that exist between the liberated glycerol backbone and free alkyl esters. Removal of glycerol is thus accomplished by allowing the processed solution to settle causing a bi-layer to form between the glycerol and alkyl esters. The glycerol or partially purified alkyl esters are then drained or pumped out respectively. The

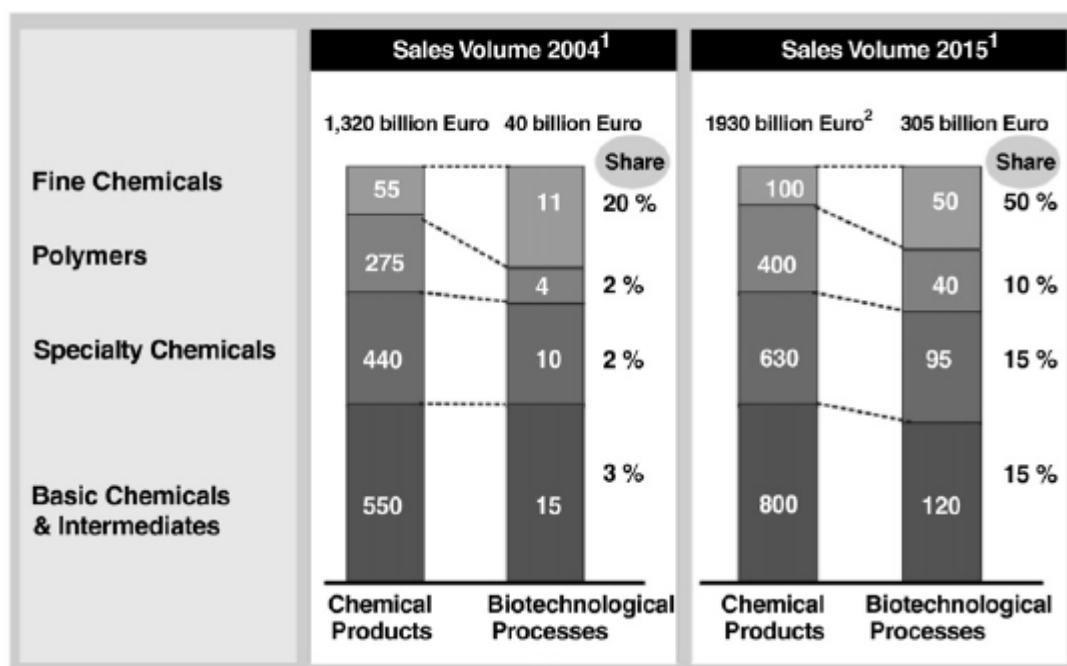
second phase of purification involves the separation of the biodiesel from unused catalyst, free metals, water, alcohol, any soaps and un-reacted free fatty acids remaining in solution. Separation is accomplished by a dry or wet washing step followed by a distillation step (Berrios and Skelton 2008). Wet washing removes any remaining glycerol, alcohol, salts, or soaps in solution as a result of their respective solubilities in water (Berrios and Skelton 2008). Further advantages of wet washing come from the high levels of purity, often 99%, and the high yield, generally 86% (Glisic and Skala 2009). The use of wet washing is a common method of purification as it is capable of removing and neutralizing contaminants effectively enough to ensure the purified biodiesel is within the EU standards for biodiesel (Berrios and Skelton 2008). Drawbacks to this method include increased processing time, cost, loss of product, inclusion of water in solution and the generation of a hazardous waste stream in the form of the contaminated water (Berrios and Skelton 2008). Dry washing is defined as replacing water with an ion exchange resin or magnesium silicate powder to neutralize impurities (Berrios and Skelton 2008). By switching to a resin or powder wet washing issues such as hazardous waste streams, increased processing time, cost and the inclusion of water in the biodiesel product are avoided while high levels of purity and yield are retained. Current dry washing methods rely on commercially available resins or powders and as such detailed information on their mechanism of action is unavailable. Further compounding the vague nature of dry washing techniques is the fact that other than advertising material little is known about their performance (Berrios and Skelton 2008). Studies by Berrios et al. testing the efficiency of dry washing resins and powder have been performed recently and found that only wet washing was capable of purifying the impure alkyl ester solution straight from the glycerol separation phase to EU standards (Berrios and Skelton 2008).

2.3 Biorefineries and glycerol as a path to success for the biofuel industry

Biofuel production is predicted to increase substantially over the coming decades (Us-Eia 2009). Limitations to the growth of the biofuels industry come from low profit margins, cost of the biomass, and constraints in infrastructure (Yazdani and Gonzalez 2007; Tyner 2008). Concerns over low profit margins, high costs, and infrastructure in conjunction with the

dependence of the industry on government policy, and subsidies have generated major concern about the economic viability of the biofuels industry at large (Yazdani and Gonzalez 2007; Tyner 2008). Therefore a means of increasing profit margins would equate to an increase in the economic viability of the biofuel industry and its continued growth. One proposed method of increasing the profit margins and thus economic viability of the biofuel industry comes from the utilization of the feed-stock and/or waste streams to co-produce a secondary high value chemical (Yazdani and Gonzalez 2007). Biorefineries have been proposed as a means of increasing the economic viability of the biofuel industry by producing high value chemical products along with biofuels. Biorefineries result in the production of typical petrochemical value added byproducts via bio-based chemistry (Van Haveren et al. 2008). Biorefineries are expected to significantly increase profit margins of the biofuels industry to a point where government policies and subsidies will no longer be necessary (Werpy 2004; Yazdani and Gonzalez 2007; Van Haveren et al. 2008).

A number of high profit value added by-products are traditionally produced through the petrochemical industry. Given the annual increasing costs of petroleum and expected shifts to a more bio-based energy system it should come as no surprise that the economic viability of traditional petrochemical by-product production is expected to decrease whereas the economic viability of feedstock or renewable based chemical by-product production is expected to increase (Van Haveren et al. 2008; Snell and Peoples 2009). As such biorefinery based value-added chemical production is expected to make a major impact in the production of bulk and platform chemicals over the next 20 years as can be seen in Figure 2.4 (Van Haveren et al. 2008).



1) Global chemical sales without pharma products but including pharma intermediates.

2) Calculation with an average growth rate of global chemical sales of 3,5% p.a.

Source: Market study of FESTEL CAPITAL

Figure 2.2 The development of the share of biotechnological processes in the sales volume of chemical by-products groups in 2004 and 2015. From Saxena et al. (2009) reprinted with permission from Elsevier.

2.3.1 Biorefineries: A definition

A biorefinery has been defined as a refinery that makes use of a fraction of the feedstock to co-produce a higher-value, small-market chemical along with the biofuel(s) (Yazdani and Gonzalez 2007). This concept of a biorefinery further extends to the use of traditional biofuel waste streams, such as unused feedstock components and traditionally defined impurities, along with a common processing operation to drive down production costs (Werpy 2004). Synergistic production has been seen for generations in the distillation and purification of crude oil to petroleum, kerosene, asphalt, and paraffin wax let alone the use of the gaseous components of crude oil distillation in the petrochemical industry. The scope of value added products that biorefineries can produce is similar to the petrochemical industry and is exceedingly large (Werpy 2004). As biorefineries are still very much in their infancy, particular focus is being applied to a small subset of chemicals of which a large number of profitable value added by-products may be produced. This small set is comprised of 12 sugar based building

block chemicals, as seen in Table 2.1, that could be the most important, most profitable, and most likely chemicals to be produced as value added by-products from biomass or waste streams (Werpy 2004). Of these sugar based building block chemicals glycerol, a common waste product of bioethanol and biodiesel production, is garnering particular interest in the biochemical and biofuels industries alike (Aldiguier et al. 2004; Yazdani and Gonzalez 2007). Glycerol is produced in excess amounts in both bioethanol and biodiesel production (Yazdani and Gonzalez 2007). In biodiesel production one mole of glycerol is produced for every three moles of alkyl ester. This equates to 1 lb of glycerol to every 10 lbs of biodiesel or in other words glycerol production is ten percent of biodiesel production (Yazdani and Gonzalez 2007). Glycerol is produced during the fermentative process of bioethanol production. The levels of glycerol found in bioethanol production are entirely dependent on the temperature of fermentation (Aldiguier et al. 2004). High rates of glycerol production have been shown to occur at 36° and 39°C (Aldiguier et al. 2004). Given that bioethanol production is strain- and temperature-dependent it can be expected that the levels of glycerol will fluctuate between different bioethanol plants. That being said, it is expected that as the biofuel industry grows so too will the production of glycerol as a waste product. It is estimated that as little as a 2% displacement of current diesel fuel with biodiesel could lead to the production of an additional 800 million pounds of glycerol (Werpy 2004). Any associated increase in the levels of glycerol production from the biofuel industry will help to lower glycerol prices while drastically increasing production (Pagliaro et al. 2009). An associated rise in biofuel consumption is seen to correlate with an inverse linear relationship between production cost of biodiesel and variations in the market price of glycerol (Pagliaro et al. 2009). All of these factors continue to enhance to reputation of glycerol as an extremely valuable by-product and possible platform chemical.

Table 2.1 Sugar based chemical byproducts of interest for biorefineries

Sugar Based Building Block Chemicals						
1,4-diacids	2,5-furan dicarboxylic acid	3-hydroxy propionic acid	aspartic acid	glucaric acid	glutamic acid	
itaconic acid	levulinic acid	3-hydroxybutyrolactone	glycerol	Sorbitol	xylitol/arabinitol	

2.3.2 Using glycerol to produce high value added by-products

Glycerol is seen as a promising platform chemical due to the associated value added by-products that it can generate through chemical or enzymatic means. It was first assumed and hoped that glycerol production from the biofuel industry could be directly converted into the production of profitable by-products. Unfortunately, glycerol produced from the biofuels industry is often contaminated with salts, water, alcohol, and other chemicals and is thus unfit for the immediate transformation into a number of by-products (Pagliaro et al. 2009). There are still a number of incredibly profitable by-products capable of being produced from contaminated or crude glycerol as can be seen from Figures 2.5 and 2.6. Glyceric acid is one such proposed by-product from the platform chemical glycerol. Glyceric acid is proposed to be produced through an aerobic oxidation pathway (Werpy 2004). Potential uses of glyceric acid are as a polylactide fiber or as a new type of polyester fiber (Werpy 2004). Succinic and propionic acid are two other potential by-products that may be produced from glycerol. Production of succinic and propionic acid is thought to be possible through the microbial conversion of glycerol by *Propionibacteria acidipropionici* and *Anaerobiospirillum succiniciproducens*, respectively (Yazdani and Gonzalez 2007). Given the use of succinic acid in the pharmaceutical industry and other value added byproducts it is of no surprise that it ranks among one of the more profitable by-products. Five chemical by-products still remain that bear particular focus for their profitability and ability to be readily converted to value added by-products.

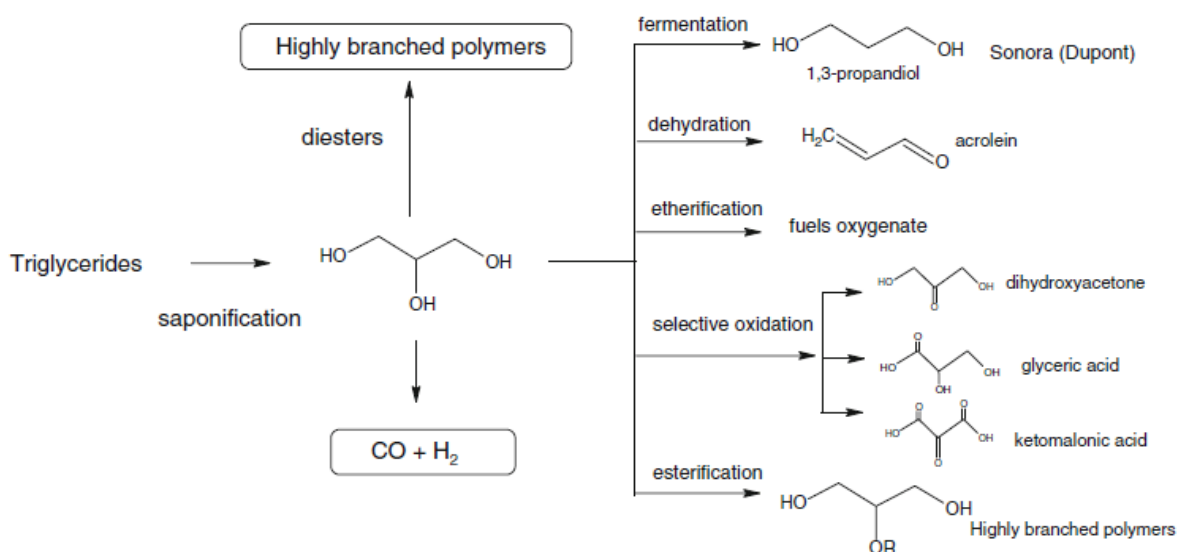


Figure 2.3 Main pathways for the utilization of glycerol
From Dimitratos et al. (2009) reprinted with permission from Springer.

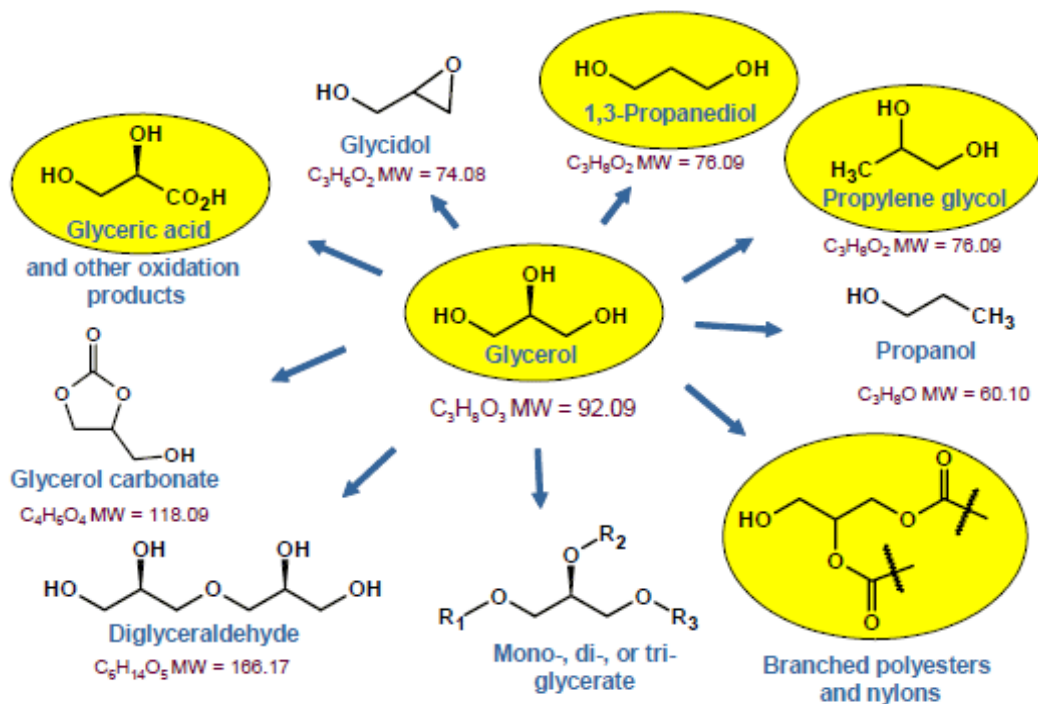


Figure 2.4 Derivatives of glycerol – Circled chemicals in commercial use at commodity-scale levels of production
Taken from U.S. Department of Energy, Energy Efficiency and Renewable Energy report - Top Value Added Chemicals From Biomass (2004) under public domain license.

Epichlorohydrin has recently started being produced from biofuel glycerol by Solvay Chemicals (Green River, WY, USA) through a proprietary process. Information regarding the Epicerol process from Solvay Chemicals is limited at best and somewhat misleading given the advertising nature of the company's website. The process as described by Solvay Chemicals makes use of glycerin from biodiesel production along with hydrochloric acid in a catalytic reaction to produce epichlorohydrin. As listed by the company the benefits include reduced number and amounts toxic chemicals and waste streams along with increased environmental benefits (Us-Eia 2009). Typical uses of epichlorohydrin as listed by Dow Chemicals are mostly as an epoxy resin, textiles, dyes and ion exchange resins. Given a price report generated by ICIS pricing in 2009, epichlorohydrin is currently trading at ~\$1,300 - \$1,400 US dollars per ton versus crude glycerin from biofuel at ~\$180 - \$250 US dollars per ton (Wilson 2009).

Acrolein is traditionally produced from a direct catalytic oxidation process (Vollenweider and Lacroix 2004). The direct catalytic oxidation process has a low yield of approximately 8% (Vollenweider and Lacroix 2004). The prominent role of acrolein is that of a pesticide in the control of weeds, algae, bacteria and mollusks. A spot price of acrolein was given as \$2,653.20 US dollars per ton in January 2010.

Acrylic acid is best known as a platform chemical given its ability to be modified and polymerized into a broad range of versatile chemicals and resins for the production of viscosity modifiers and flocculants. Acrylic acid may be produced from the oxidation of 3-hydroxypropionaldehyde (3-HPA) (Vollenweider and Lacroix 2004). Traditional methods of acrylic acid production stem from the gas-phased catalytic oxidation of the petrochemical product propylene. Demand for acrylic acid rose sharply from 1980 to 2002 and is expected to continue to rise to 4 million tons by 2009 (Vollenweider and Lacroix 2004). Spot prices for acrylic acid have been placed at \$1,300 - \$1,500 US dollars per ton as of November 2009 (Icis.Com 2009). Given that traditional methods are inefficient alternative microbial approaches are being explored (Vollenweider and Lacroix 2004).

The chemical 1,3-PDO has been traditionally produced via the Degussa and Shell process. The Degussa and Shell process convert propylene into acrolein via a catalytic solution phase hydration and from ethylene oxide via a hydroformylation and subsequent hydrogenation

respectively (Saxena 2009). Until recently the global demand for 1,3-PDO was quite small. However, given the introduction of a new polyester in 1995 by Shell based on 1,3-propanediol demand has been increasing rapidly. The new polyester, polytrimethylene terephthalate (PTT) has a number of excellent properties and potential applications. DuPont has a number of similar PTT based polymers with similar properties and potential applications. Of recent note is the use of microorganisms in the production of 1,3-PDO. The use of 1,3-PDO in the production of polyesters is seen as one of the most promising and profitable by-products available. Given the polyester market is in the neighborhood of ~2-3 billion lb/yr and nylons are in the 9 billion lb/yr market, the potential level of production is enormous (Werpy 2004). Further, given prices ranging from ~\$1,700 – \$4,400 US dollars per ton versus crude glycerol at ~ \$180 - \$250 US dollars per ton, the potential profit margin is large.

A precursor to 1,3-PDO (3-HPA) is the final major commercial by-product expected to be readily converted from glycerol and be extremely profitable. The chemical 3-hydroxypropanediol is manufactured in a similar fashion to that of 1,3-PDO as the Degussa and Shell process are the primary modes of conversion of glycerol to 3-HPA. Unfortunately the Degussa and Shell processes are inefficient modes of conversion. In the Shell process an organic phase separation is required to extract and purify the 3-HPA from 1,3-PDO. In the Degussa process the yield of 3-HPA is questionable as no production data was found to be available (Vollenweider and Lacroix 2004). As such alternative methods of producing 3-HPA have garnered great deals of attention with microbial conversion being among the most promising. Given 3-HPA is a pre-cursor to 1,3-propionaldehyde many of the same markets and business opportunities may be exploited by 3-HPA as well. That being said, as a precursor chemical 3-HPA has a number of supplementary market options. One such option is in the production of acrylic acid. Acrylic acid ranges in price from ~\$1,300 - \$1,500 US dollars per ton as previously discussed (Icis.Com 2009).

2.4 Microbial production of 1,3-propanediol

The microbial production of 1,3-PDO is known to occur in a specific set of Gram-positive and negative bacteria. This set includes enteric acid bacteria, propionic acid bacteria, clostridia

and lactobacilli (Daniel et al. 1998). Increased cost and the associated toxicity of 1,3-PDO production from traditional chemical synthesis has led to an increased interest in microbial species capable of producing 1,3-PDO (Biebl 1999; Mu 2006). Chemical synthesis of 1,3-PDO is known to occur through the Degussa and Shell process. Drawbacks of the Degussa process include the generation of acrolein a toxic product, an expensive separation phase to isolate 1,3-PDO, and low yields of 43% (Vollenweider and Lacroix 2004). The Shell process has increased yields of 80% but is limited by the need for a high pressures environment and an expensive separation phase to isolate 1,3-PDO (Vollenweider and Lacroix 2004). As a result, exploration into microbiological solutions capable of facilitating the conversion of glycerol to 3-HPA and 1,3-PDO are being explored. The yield of 1,3-PDO produced will depend on the micro-organism chosen as well as the process conditions (Biebl 1999). Two of the primary candidates for the production of 1,3-PDO from a microbial species are *Citrobacter freundii* and *Klebsiella pneumoniae* due to their substrate tolerance, yield, and productivity (Saxena 2009). However, the uses of *C. freundii* and *K. pneumoniae* in the production of 1,3-PDO require special safety precautions given their status as opportunistic pathogens. Additional difficulties associated with low yield and productivity from the microbial production of 1,3-PDO have led to studies involving the optimization of process parameters and the generation of genetically engineered strains (Saxena 2009). The generation of genetic engineered species and the optimization of process parameters both focus on optimizing the metabolic pathways associated with the conversion of glycerol to 1,3-PDO to maximize yield.

The production of 1,3-PDO occurs through the reduction of glycerol to 3-HPA which is subsequently converted to 1,3-PDO (Johnson and Lin 1987; Talarico and Dobrogosz 1989; Talarico et al. 1990; Talarico and Dobrogosz 1990; Luthi-Peng et al. 2002; Baeza-Jimenez et al. 2011). The enzymes responsible for the conversion of glycerol to 1,3-PDO are glycerol dehydratase (EC 4.2.1.30) and 1,3-propanediol oxidoreductase, sometimes referred to as 1,3-propanediol dehydrogenase (EC 1.1.1.202) (Johnson and Lin 1987; Talarico and Dobrogosz 1989; Talarico et al. 1990; Talarico and Dobrogosz 1990; Luthi-Peng et al. 2002; Baeza-Jimenez et al. 2011). All known glycerol dehydratase containing bacteria are co-enzyme B12-dependent (Class II of co-enzyme B12 containing enzymes) with the exception of *Clostridium glycolicum* (Daniel et

al. 1998). This group of enzymes is defined as enzymes catalyzing heteroatom elimination reactions (Toraya 2000). The basic function of glycerol dehydratase is to remove an oxygen and hydrogen in the form of water from its substrate, as in the formation of 3-HPA. Glycerol dehydratase is known to convert the substrates glycerol, 1,2-propanediol and 1,2-ethanediol to their corresponding aldehydes (Daniel et al. 1998). The reaction responsible for this conversion is known to proceed by a mechanism involving coenzyme B12 as an essential cofactor (Daniel et al. 1998). The generation of a highly-reactive free-radical is necessary for catalysis. The generation of a highly reactive free-radical leads to three potential problems for the enzyme: (i) generation of the radical must be confined to the active site (ii) control of the reactive intermediates is vital (iii) the when and how of reactivation from undesired side reactions must be controlled (Toraya 2000). In glycerol dehydratase the radical is formed from adenosylcobalamin (AdCo) that is bound to the enzyme by the imidazole of a histidine residue (Daniel et al. 1998). The binding of the AdCo in the active site activates the homolytic cleavage of the AdCo Co-C bond to form cob(II)alamin and an adenosyl radical (Daniel et al. 1998; Toraya 2000). Cleavage of the AdCo Co-C bond has been shown to trigger the reaction by activating the substrates through abstraction of a hydrogen atom (Toraya 2000). After abstracting the hydrogen atom a substrate derived radical is formed which rearranges to a product radical by a hydroxyl group transfer from C-2 to C-1 (Daniel et al. 1998). The product radical then takes a hydrogen atom from a 5'-deoxyadenosine to generate the final product and regenerate the co-enzyme (Daniel et al. 1998). This reaction can be seen in Figure 2.5.

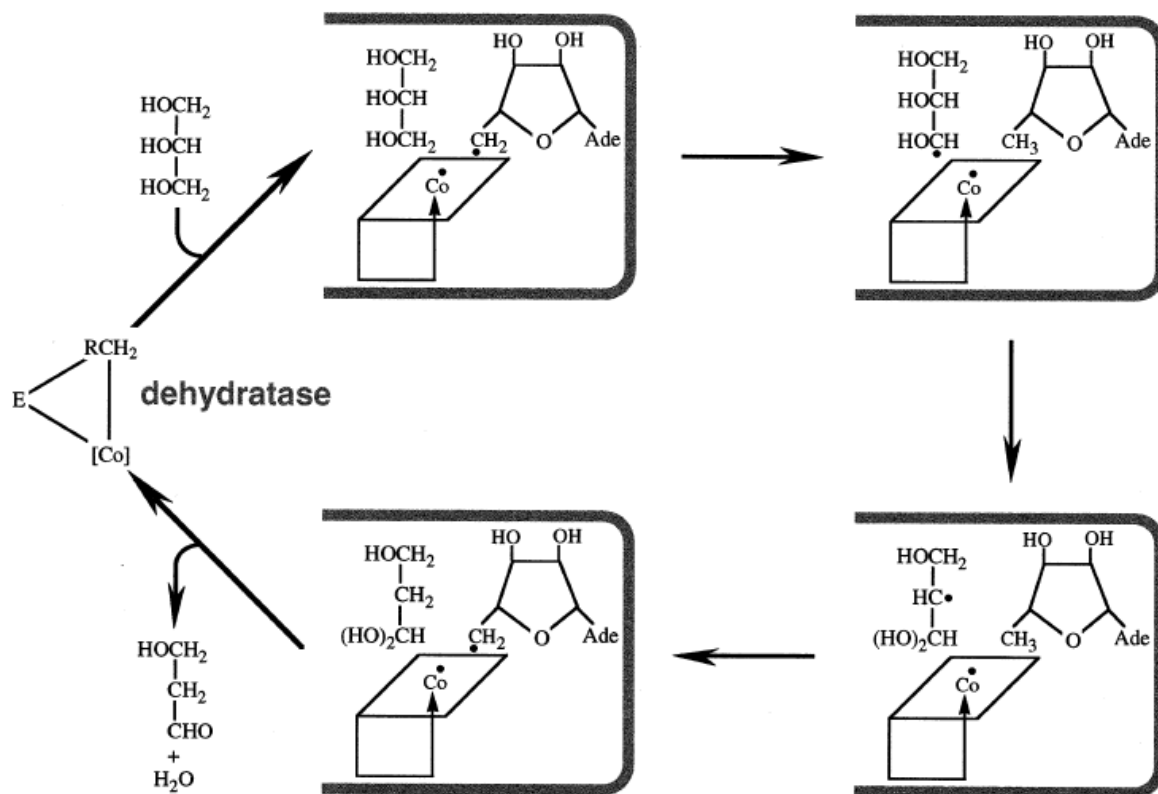


Figure 2.5 Mechanism of glycerol dehydratase reaction. The co-enzyme B12 is depicted in the base-on form. ADE, adenine; RCH₂, adenosyl; [Co], cobalamin; E, enzyme. Reprinted from Daniel et al. (1998) with permission from John Wiley and Sons (Daniel et al. 1998)

By ensuring that the generation and subsequent sequential reaction of the free radical is mediated via a coenzyme to substrate to product to coenzyme fashion glycerol dehydratase ensures the free radical is formed specifically within the active site and the intermediates are tightly regulated. Unwanted side reactions are inevitable and lead to a mechanism-based suicide inactivation of the enzyme. Inactivation occurs by irreversibly cleaving the Co-C bond forming a 5'-deoxyadenosine and alkylcobalamin species. The enzyme then tightly binds the modified co-enzyme ensuring containment of the radical. Thus glycerol dehydratase ensures the entrapment of any possible free radicals and contains a specific mechanism to discontinue the reaction if unwanted side reactions occur. However, the pathway of glycerol breakdown has almost exclusively been studied in *C. freundii* and *K. pneumoniae*. Information in regards to the pathway of glycerol breakdown in other species such as *L. reuteri* remains limited. This can be

seen in structural studies as a predominantly large portion of the focus is on the *K. pneumoniae* glycerol dehydratase enzyme composed of three subunits in the following configuration $\alpha_2\beta_2\gamma_2$. Additionally, the concentrations of vitamin B12 required for glycerol dehydratase enzymes from different species appears to vary. The Gram-positive bacteria *L. reuteri* has shown a high K_m value for vitamin B12 (0.3 μ M) and thus requires very little of the compound. Additionally, *L. reuteri* has been found to be able to convert up to 85% of glycerol to 3-HPA in dilute glycerol solutions. Irrespective of the choice of organism or process parameter chosen it has become clear that there is still a significant amount of work to be done to find/generate an optimized microbe capable of producing 1,3-PDO to industrial levels in a cost effective manner.

2.5 Objective and project overview

The hypothesis of this study is that the production of 1,3-PDO from *Lactobacillus panis* depends on the supply of glycerol, glucose, and on the physical conditions. Further, the optimization of these conditions will give insights into the metabolic pathways of this strain. The overall objective of this study was to optimize the 1,3-PDO production conditions through examination of chemical and physical conditions, and characterization of glycerol converting enzymes.

The major objectives of this project were:

- A) Optimization and characterization of 1,3-PDO production from *L. panis* PM1
 - a. To determine the effect of glucose concentration, glycerol concentration, inoculum size, temperature, aeration, pH and the choice of carbon source on 1,3-PDO production; and
 - b. To gain insight into the metabolic control of 1,3-PDO production in *L. panis* PM1.
- B) Fed-Batch production of 1,3-PDO from *L. panis* PM1
 - a. To determine the ability of the *L. panis* PM1 isolate to produce 1,3-PDO in a fed-batch culture; and
 - b. To increase the duration and mole quantity of 1,3-PDO production; and
 - c. To gain insight into the metabolic control of 1,3-PDO production in *L. panis* PM1.
- C) Cloning and characterization of a NAD⁺-dependent oxidoreductase from *L. reuteri* DSM 20016
 - a. To clone and express an active recombinant form of the 1,3-PDO NAD⁺-dependent oxidoreductase from *L. reuteri* DSM 20016; and
 - b. To determine kinetic values for NAD⁺/NADH and 1,3-PDO for the enzyme.

3. STUDY 1 - OPTIMIZATION AND CHARACTERIZATION OF 1,3-PROPANEDIOL PRODUCTION FROM A NOVEL *LACTOBACILLUS PANIS* PM1 ISOLATE

3.1 Abstract

Interest in the aliphatic carbon compound 1,3-propanediol (1,3-PDO) has risen due to its role in the production of biodegradable polymers. Our research group previously reported the identification of a novel *Lactobacillus panis* PM1 isolate capable of converting glycerol to 1,3-PDO. Process parameters have been shown to effect the production of 1,3-PDO (Ahrens et al. 1998; Chen et al. 2003; Doleyres et al. 2005; Durnin et al. 2009; Vanajakshi and Annapurna 2011). Parameters such as the concentration of glucose, glycerol, inoculum size, temperature, aeration, pH and the choice of carbon source were examined to identify the optimal conditions for 1,3-PDO production. Data collected by proton NMR and commercially available assays indicated that the concentration of glycerol and pH played a primary role in determining the final concentration of 1,3-PDO. Optimal conditions for the production of 1,3-PDO were determined to be a carbon limited culture, defined as: below 50 mM glucose, grown at 37°C without agitation containing glycerol in the range of 150 – 250 mM at an elevated pH of 9 – 10. Factors such as inoculum size and temperature were not shown to influence the production of 1,3-PDO as expected. We believe that the data collected further demonstrates that the bottleneck for 1,3-PDO production lies in maintaining an appropriate pool of NAD⁺/NADH while mitigating the negative effects associated with the accumulation of toxic by-products.

3.2 Introduction

The rising costs associated with the production of petrochemicals, and the stagnation of new capital investment in the chemical industry in the United States, is aiding the growth and innovation of alternative petrochemical production methods (Snell and Peoples 2009). Interest in alternative means of petrochemical production represents a boon to the bio-based industry as a number of chemical platforms can be derived from renewable biological sources. In particular, the bioplastic polytrimethylene terephthalate (PTT) has received increased attention over the past few years. However, until recently, interest in PTT was traditionally academic due to the low availability and high associated cost for the required intermediate, 1,3-propanediol (1,3-PDO) (Biebl 1999). An increase in 1,3-PDO production in combination with a decrease in 1,3-PDO price has aided the realization of a PTT-based plastic being produced at industrial levels (Zhang 2004). Dupont™ is currently producing a PTT-based plastic under the brand name Sorona®. Due to industrial interest 1,3-PDO is transitioning to a commodity, and potentially a bulk chemical, with demand already exceeding 100 million pounds per year (Saxena 2009). As 1,3-PDO is also used in the textile industry, production of foods, lubricants, packaging, and medicinal usage, the demand for 1,3-PDO will likely continue to rise.

The chemical 1,3-PDO is a linear aliphatic glycol with two terminal hydroxyl groups. Traditional production of 1,3-PDO occurs through chemical catalysis. Chemical catalysis involves the hydration of acrolein to 1,3-PDO. Alternatively, 1,3-PDO can be generated from the hydroformulation of ethylene oxide to 3-HPA followed by a hydrogenation. An alternative to the chemical synthesis of 1,3-PDO exists in the ability of various microorganisms to produce 1,3-PDO through the fermentation of glycerol. Interest in the microbial production of 1,3-PDO is due to its relative ease and the lack of toxic byproducts when compared to chemical synthesis (Mu 2006). Further, glycerol is produced as a waste product in the biofuel industry and represents, not only an economically viable substrate in the production of 1,3-PDO, but an entirely renewable one (Saxena 2009). As a result, interest in identifying, characterizing, and optimizing the production of 1,3-PDO from microbes has become a topic of interest.

The ability of bacteria to produce 1,3-PDO has been documented and observed since 1881 by August Freund (Saxena 2009). The bacteria capable of generating 1,3-PDO do so through

glycerol fermentation and have been identified as the enterobacteria *Klebsiella*, *Enterobacter*, *Citrobacter*, *Lactobacillus*, and *Clostridia* (Biebl 1999; Saxena 2009). To date *L. buchneri*, *L. reuteri*, *L. hilgardii*, and *L. diloovorans* are the reported members of the genus *Lactobacillus* capable of producing 1,3-PDO from the fermentation of glycerol (Biebl 1999). The fermentation of glycerol leads to the production of 3-hydroxypropionaldehyde (3-HPA) by a coenzyme B12-dependent glycerol dehydratase, which is then subsequently converted to 1,3-PDO by a 1,3-PDO NAD⁺-dependent oxidoreductase (Johnson and Lin 1987; Talarico et al. 1990; Ahrens et al. 1998; Luthi-Peng et al. 2002; Baeza-Jimenez et al. 2011). The anaerobic fermentation of glycerol in *Enteric* bacteria has been proposed as an auxiliary pathway to maintain the redox-balance of the organism (Gonzalez et al. 2008). The NAD/NADH ratio has been shown to be important in determining the production of 1,3-PDO in other bacterial producers (Barbirato et al. 1996). Therefore, factors affecting the redox-balance such as pH, glycerol concentration, and the production of fermentation gasses such as hydrogen and carbon dioxide are thought to affect glycerol fermentation (Gonzalez et al. 2008; Tobajas et al. 2009; Bauer et al. 2010; Vanajakshi and Annapurna 2011).

We have identified a novel *L. panis* PM1 isolate from thin stillage capable of producing 1,3-PDO by the fermentation of glycerol (Khan et al. 2012). This strain is advantageous to the *Enteric* bacteria since *Lactobacillus* species are generally non-pathogenic. Thus, we believe that our *L. panis* PM1 strain can contribute to a less-hazardous industrial fermentation of glycerol. The aim of the present study is to optimize the ability of the novel *L. panis* to produce 1,3-PDO from glycerol. Studies of other 1,3-PDO producing strains have demonstrated that process parameters can vastly effect the production of 1,3-PDO (Ahrens et al. 1998; Chen et al. 2003; Doleyres et al. 2005; Yazdani and Gonzalez 2008; Durnin et al. 2009; Baeza-Jimenez et al. 2011; Vanajakshi and Annapurna 2011). Our previous study (Khan et al. 2012) showed the effect of various conditions on the growth and survival of the *L. panis* PM1 and that it was capable of generating 1,3-PDO under certain conditions, i.e., starvation conditions with low oxygen availability. In the present study, we hypothesize that a further examination of the conditions will aid in determining and defining the optimal conditions for the production of 1,3-PDO from glycerol by our novel *L. panis* PM1 isolate.

Objectives of the study were:

- A) To determine the effect of glucose concentration, glycerol concentration, inoculum size, temperature, aeration, pH and the choice of carbon source on 1,3-PDO production by *L. panis* PM1;
- B) To optimize production of 1,3-PDO from *L. panis* PM1 in a static culture; and
- C) To gain insight into the metabolic control of 1,3-PDO production from *L. panis* PM1.

3.3 Materials and Methods

3.3.1 Chemicals and instruments

A modified MRS media (mMRS) was generated and composed of 5 g yeast extracts, 10 g tryptone, 5 g meat extract, 2 g K_2HPO_4 , 2 g ammonium citrate, 5 g sodium acetate, 100 mg $MgSO_4 \cdot 7H_2O$, 50 mg $MnSO_4$, and a defined concentration of glucose and glycerol per litre. Free Glycerol Determination Kit (cat# FG0100) and Glucose (HK) Assay kit (cat# GAHK-20) were purchased from Sigma-Aldrich (Oakville, ON, Canada). NMR analysis was performed on a 500 MHz machine (Model AMX 500-MHz, NMR Bruker, Mississauga, ON) at the Saskatoon structural sciences center (Saskatoon, SK, Canada). Other chemicals used in this research were ACS-grade or better and purchased from VWR international (Mississauga, ON, Canada).

3.3.2 Determination of *L. panis* PM1 cellular growth, 1,3-PDO and major metabolite concentrations

Cellular growth was determined by observing the changes in the absorbance at an optical density at 600 nm (OD_{600}) in a Beckman DU800 spectrophotometer. Samples were performed in triplicate and for samples with an OD_{600} reading over 1.00, a one in ten dilution was made in H_2O to ensure a more accurate OD reading.

Concentrations of glucose and glycerol were determined by the Free Glycerol Determination Kit and Glucose (HK) Assay kit following manufactures guidelines. Samples for these assays were prepared using centrifugation at 12,000 x *g* in a bench top centrifuge and the supernatant was passed through a 0.45- μ M filter to remove any remaining bacterial cells and large particulate materials.

Concentrations of 1,3-PDO and other major metabolites, e.g. ethanol, lactic acid, and acetic acid, were determined by proton NMR following previously established protocol (Ratanapariyanuch et al. 2011). Samples were centrifuged at 12,000 x*g* in a benchtop centrifuge for 5 minutes and the supernatant was collected. The collected supernatant was passed through a 0.45- μ M filter to remove particulate material. A 500- μ L aliquot from the filtered supernatant was mixed with 50- μ L of deuterium oxide (D_2O) and 50- μ L of 0.54 M dimethylformamide (DMF)

solution. Concentrations of 1,3-PDO, ethanol, acetic acid, and lactic acid were quantitatively determined in comparison to the known molar concentration of the internal DMF standard and the analytes associated proton to signal ratio (Ratanapariyanuch et al. 2011).

3.3.2 Metabolite concentrations during initial 1,3-PDO production

A culture based method was employed to determine the kinetics of 1,3-PDO production following the methodology described by Luthi-Peng et al. (Luthi-Peng et al. 2002). This method involves the generation of a starved resting cell mass and the subsequent re-suspension of that cell mass in a defined media at a defined cell density in order to simulate secondary metabolic growth under defined conditions. The growth of *L. panis* PM1 was conducted as follows unless otherwise stated; *L. panis* PM1 was grown in 50 mL of MRS (DIFCO, Sparks, MD, USA) broth in a 50-mL conical tube at 37°C for 24 hours. The culture was conducted under micro-aerobic conditions where no oxygen was introduced to the culture by agitation or other means but no oxygen was removed either. Cells were harvested by centrifugation at 11,200 x *g* in an SS-34 rotor in a Sorval high-speed centrifuge at 4°C for 20 minutes. The cell pellet was re-suspended and washed with deionized H₂O twice. Cell lysates were decanted and the sterile washed cell mass was re-suspended in one tenth of the original volume in mMRS broth containing 100 mM glucose and 200 mM glycerol. The 5-mL re-suspended culture was added to a fresh 45 mL mMRS culture containing 100 mM glucose and 200 mM glycerol in a 50-mL conical tube. The inoculated culture was grown at 37°C without agitation under micro-anaerobic conditions. Headroom in the conical tube was kept to a minimum to reduce the availability of molecular oxygen. At various time points, a 1-mL sample was collected and the concentration of glucose, glycerol, 1,3-PDO, and various other major metabolites were determined by proton NMR as previously described.

3.3.3 Examination of carbon sources capable of producing 1,3-PDO

The novel *L. panis*, as indicated by a bioMérieux API 50 CHL carbohydrate kit C, was capable of metabolizing glucose and xylose (Khan et al. 2012). To determine the optimal carbon source for the production of 1,3-PDO cultures were grown as previously described with the

following amendments. Individual cultures were re-suspended in a final volume of 50 mL of mMRS media containing 50 mM of glucose or xylose and 150 mM glycerol in a 50-mL conical tube. Unsupportive sucrose and no additional sugar were used as a negative reference. Samples were incubated at 37°C without agitation. Samples (1 mL) were taken at 0, 3, 7, 24, 48, 72, and 96 hours. Samples were analyzed by proton NMR for 1,3-PDO and major metabolites as previously described. The effects of glucose concentration were also determined by culturing in 5, 10, 20, 30, 40, and 50 mM glucose mMRS media and determining the change in ethanol and 1,3-PDO production.

3.3.4 Effects of culture atmosphere conditions on 1,3-PDO production

The overnight culture was re-suspended in a total volume of 100-mL mMRS media containing 100 mM glycerol and 50 mM glucose. The 100-mL sample was split into two 50-mL conical tubes. One re-suspended sample was placed in a shaking incubator at 37°C and agitated at 150 rpm resulting in an aerobic environment. The second re-suspended sample was placed in the same incubator without agitation at 37°C resulting in a microaerobic environment. Samples (1 mL) from the agitated and non-agitated cultures was taken at 0 and 96 hrs and assayed for 1,3-PDO and major metabolites by proton NMR as previously described.

3.3.5 Effects of initial glycerol concentration on 1,3-PDO production

The overnight culture was re-suspended to a final volume of 50-mL mMRS media containing 50 mM glucose and concentrations of glycerol ranging from 0.025 to 1 M in a 50-mL conical tube. Samples were incubated at 37°C without agitation. Samples (1 mL) were taken at 0, 24, and 48 hours. Samples were analyzed for 1,3-PDO and major metabolites by proton NMR as previously described. The conversion efficiency was determined by dividing the concentration of 1,3-PDO by the concentration of glycerol added.

3.3.6 Effects of initial pH on 1,3-PDO production

The overnight culture was re-suspended in an mMRS media containing 100 mM glucose and 300 mM glycerol. The pH was adjusted for individual samples from a pH of 5 to 10 using 1 N

HCl and 1 M NaOH with a VWR symphony SB70p pH meter and monitored at 0 and 96 hours. Samples were incubated at 37°C without agitation. A 1-mL sample was taken at 0, 24, 48, 72, and 96 hours. Samples were assayed for 1,3-PDO and major metabolites by proton NMR as previously described.

3.3.7 Effects of temperature on 1,3-PDO production

The overnight culture was re-suspended in an mMRS media containing 50 mM glucose and 150 mM glycerol. Samples were incubated at 15, 20, 25, 30, and 37°C without agitation. Temperatures above 40°C were not tested due previous studies indicating an inability of the organism to maintain adequate growth above 42°C. A 1-mL sample was taken at 0, 24, 48, 72, and 96 hours. Samples were assayed for 1,3-PDO and major metabolites by proton NMR as previously described.

3.3.8 Effects of initial cell density on 1,3-PDO production

The overnight culture was re-suspended in an mMRS media containing 50 mM glucose and 150 mM glycerol. Samples were re-suspended to a final OD₆₀₀ of 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 as determined by a Beckman Coulter DU800 spectrophotometer. Samples were incubated at 37°C without agitation for 96 hours. A 1-mL sample was taken at 0, 24, 48, 72, and 96 hours. Samples were assayed for 1,3-PDO and major metabolites by proton NMR as previously described.

3.4 Results

3.4.1 Metabolite concentrations during initial 1,3-PDO production

Glucose was consumed after 12 hours. Cellular growth corresponded with the availability of glucose as after 12 hours no significant change in growth was observed (Figure 3.1). The results also showed that the decrease in glucose concentration corresponded with increasing ethanol and 1,3-PDO production. Initial 1,3-PDO production occurred at 4 hours with the largest increase in 1,3-PDO occurring 8 to 12 hours post inoculation (Figure 3.1, Table 3.1 and 3.2). The production of 1,3-PDO occurred as concentrations of glucose dropped below 50% of the initial concentration (i.e., less than 50 mM). The concentration of glucose in the mMRS media was reduced to 50 mM from 100 mM to determine the effects of glucose concentration on 1,3-PDO production. The data showed low levels, 5.2 ± 0.5 and 5.6 ± 0.6 mM respectively, of 1,3-PDO within the first two hours (1 and 2). However, after 3 hours, the amount of 1,3-PDO jumped to 10.9 ± 0.4 mM and it reached 14.3 ± 0.7 mM after 4 hours incubation. Xylose and the two control carbon sources (sucrose and no sugar) did not support considerable amounts of 1,3-PDO production (Figure 3.2). The results further support the correlation of 1,3-PDO production to low concentrations of available glucose. Concentrations of glucose ranging from 5 to 50 mM were tested to determine the optimal concentration of glucose for the conversion of glycerol to 1,3-PDO. The results indicated that there is no statistical difference in the production of 1,3-PDO for concentrations of glucose at and below 50 mM. An average concentration of 1,3-PDO of 74.5 ± 1.0 mM was produced for cultures grown with 5 to 50 mM glucose over a 24-hour period. While glucose concentrations in a range of 5 to 50 mM did not affect the production of 1,3-PDO, increasing concentrations of glucose had a positive correlation to increased ethanol production. Cultures grown in 10 mM glucose produced 12.3 ± 0.1 mM ethanol whereas cultures grown in 50 mM glucose produced 36.5 ± 0.5 mM ethanol.

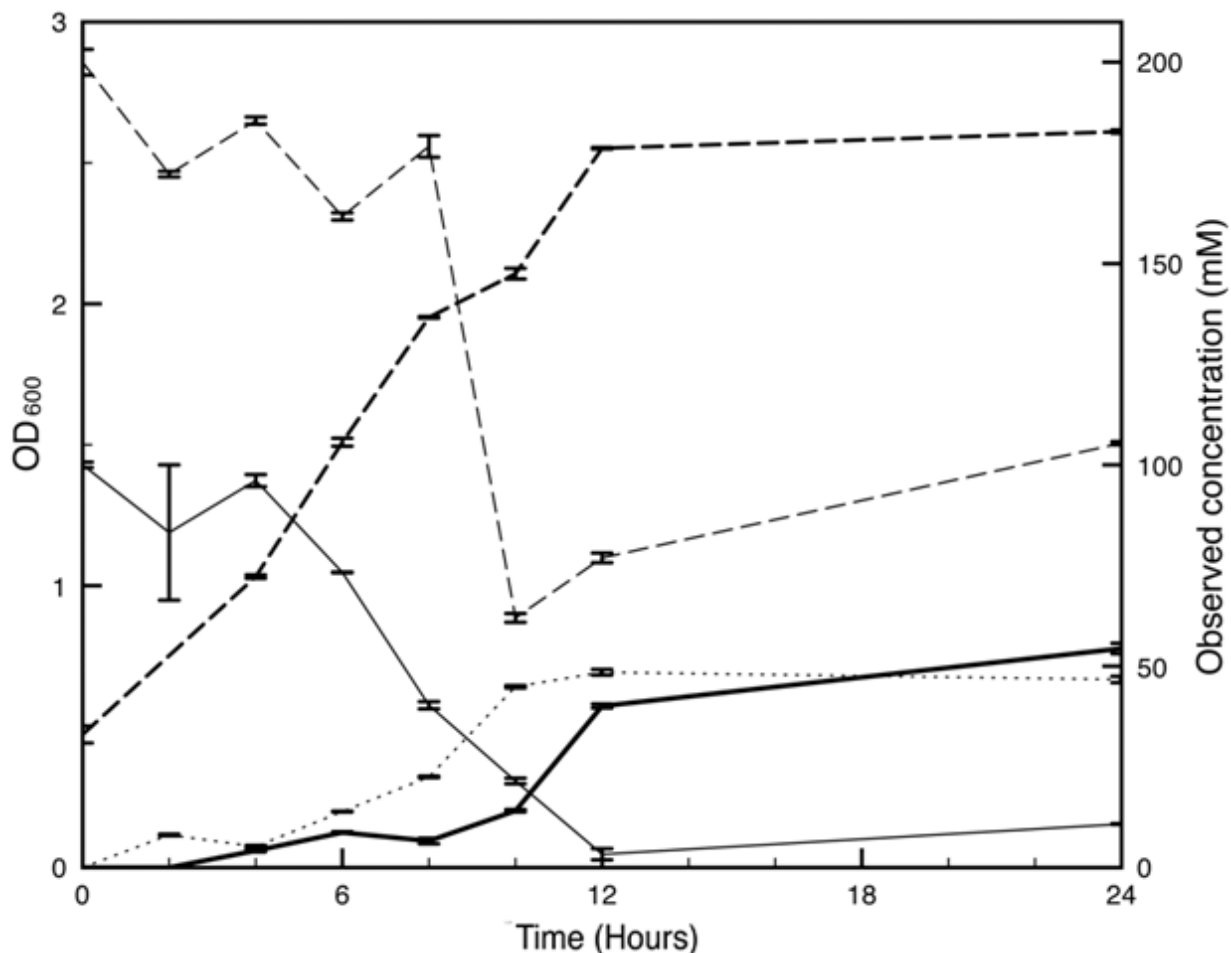


Figure 3.1 The change in metabolite concentration (1,3-PDO, glucose, glycerol, and ethanol) and growth for a culture of *L. panis* re-suspended in mMRS over 24 hours at 37°C without agitation

A culture of *L. panis* grown in MRS and re-suspended in an mMRS media was monitored over 24 hours for changes in media composition and metabolite formation. Changes in the concentration of glucose (-----), and glycerol (.....) were determined by the free glycerol and glucose (HK) assay kit from Sigma Aldrich. Cellular growth (-----) was monitored by observing changes at an OD₆₀₀ whereas ethanol (-----) and 1,3-PDO (——) concentrations were determined by molar comparison to an internal DMF standard.

3.4.2 Production of 1,3-propanediol by *L. panis* PM1 utilizing various carbon sources

The effects of various carbon sources on the ability of the novel *L. panis* isolate to produce 1,3-PDO were examined. Among the conditions examined only glucose showed considerable amounts of 1,3-PDO. Glucose produced 80 ± 0.1 mM of 1,3-PDO within 24 hours, and the production stayed at a high level between 80 ± 0.1 mM to 106.0 ± 2.3 mM for 96 hours.

Maximum OD₆₀₀ and 1,3-PDO concentrations of 1.45 ± 0.01 , 0.73 ± 0.01 mM and 106.0 ± 2.3 , 4.5 ± 0.3 mM were achieved for the samples containing glucose and with no carbon source respectively. Additionally, glucose was shown to be the optimal carbon source to obtain cell mass and other tests did not give significant increase in the cell density.

3.4.3 Effects of culture atmosphere conditions on 1,3-PDO production

Aeration negatively impacted the production of 1,3-PDO and ethanol in both absolute amount of production and conversion efficiency (Table 3.4). The production of 1,3-PDO and ethanol was reduced when the samples were aerated. Meanwhile the production of acetic acid was increased under aerobic conditions from 64.8 ± 0.01 mM to 85.3 ± 0.03 mM.

Table 3.1 Re-suspension of *L. panis* PM1 in mMRS (100 mM glucose and 200 mM glycerol) and the associated production of 1,3-PDO, ethanol, OD₆₀₀, and glucose consumption (N = 2)

Study 1				
Time (hours)	Glucose (mM)	1,3-PDO (mM)	Ethanol (mM)	OD ₆₀₀
24	10.8 ± 0.1	54.4 ± 1.3	46.7 ± 0.8	2.61 ± 0.01
12	3.3 ± 1.4	40.1 ± 0.5	48.5 ± 0.7	2.55 ± 0.01
10	21.5 ± 0.7	14.1 ± 0.3	44.9 ± 0.3	2.11 ± 0.02
8	40.3 ± 0.9	6.6 ± 0.1	22.5 ± 0.2	1.95 ± 0.01
6	73.3 ± 0.1	8.7 ± 0.1	13.9 ± 0.1	1.51 ± 0.01
4	96.2 ± 1.5	4.2 ± 0.1	5.2 ± 0.3	1.03 ± 0.01
2	83.1 ± 0.9	0.0 ± 0.1	8.1 ± 0.2	N/A
0	100.0 ± 0.7	0.0 ± 0.1	0.0 ± 0.1	0.47 ± 0.03
Study 2				
Time (hours)	Glucose (mM)	1,3-PDO (mM)	Ethanol (mM)	OD ₆₀₀
14	18.6 ± 0.2	3.3 ± 1.0	22.9 ± 0.1	2.73 ± 0.01
13	23.4 ± 0.3	1.8 ± 0.1	21.7 ± 0.1	2.32 ± 0.01
12	36.3 ± 0.1	1.2 ± 0.1	21.2 ± 0.2	1.85 ± 0.11
11	38.9 ± 0.1	0.0 ± 0.1	19.1 ± 0.1	1.71 ± 0.01
10	47.3 ± 0.1	0.0 ± 0	15.4 ± 0.1	1.54 ± 0.01
9	46.9 ± 0.1	0.0 ± 0	13.2 ± 0.1	1.41 ± 0.01
0	100.0 ± 0.1	0.0 ± 0	0.0 ± 0	0.72 ± 0.02

Change in glucose, glycerol, OD₆₀₀, and the concentration of 1,3-PDO were monitored and determined at various time points over 24 hours. Studies were performed in duplicate and the samples were read in triplicate.

Table 3.2 Production of 1,3-PDO over 24 hours in a culture of *L. panis* grown in MRS and resuspended in mMRS containing 100 mM glucose and 200 mM glycerol

1,3-PDO Production over Time (mM)						
Time (hours)	Glucose (mM)					
	0	5	10	20	30	50
0	0.0	0.0	0.0	0.0	0.0	0.0
2	20.3 ± 0.3	24.3 ± 0.8	43.0 ± 0.5	31.7 ± 0.8	29.2 ± 0.3	27.2 ± 0.1
4	35.0 ± 1.6	29.5 ± 8.5	48.9 ± 2.1	49.0 ± 0.5	46.7 ± 1.0	51.6 ± 4.5
6	49.2 ± 0.7	37.6 ± 5.6	63.4 ± 1.5	54.0 ± 2.8	60.7 ± 0.5	72.2 ± 0.4
8	61.8 ± 0.6	51.8 ± 12.3	70.8 ± 1.7	70.9 ± 2.4	64.0 ± 0.4	75.6 ± 5.2
24	72.0 ± 1.4	85.0 ± 16.5	72.3 ± 0.3	78.0 ± 1.7	75.7 ± 1.7	101.0 ± 6.0
						74.3 ± 0.3

Table 3.3 Production of ethanol over 24 hours in a culture of *L. panis* grown in MRS and resuspended in mMRS containing 100 mM glucose and 200 mM glycerol

Ethanol Production over Time (mM)						
Time (hours)	Glucose (mM)					
	0 mM	5 mM	10 Mm	20 mM	30 mM	50 mM
0	0.0	0.0	0.0	0.0	0.0	0.0
2	2.1 ± 0.1	4.2 ± 0.2	4.4 ± 0.1	2.9 ± 0.1	3.0 ± 0.0	3.0 ± 0.1
4	9.8 ± 0.1	7.3 ± 1.6	11.2 ± 0.2	11.5 ± 0.1	12.5 ± 0.1	13.8 ± 0.3
6	9.7 ± 0.1	15.9 ± 3.5	11.3 ± 0.1	14.7 ± 0.1	21.8 ± 0.3	19.4 ± 0.1
8	9.8 ± 0.0	17.3 ± 2.7	12.0 ± 0.2	13.9 ± 0.8	18.8 ± 2.6	21.2 ± 0.2
24	10.9 ± 0.8	24.4 ± 1.8	12.3 ± 0.1	18.5 ± 0.3	21.1 ± 0.2	34.5 ± 0.0
						36.5 ± 0.5

Change in glucose and the concentration of ethanol were monitored and determined at various time points over 24 hours. Studies were performed in duplicate and the samples were read in triplicate.

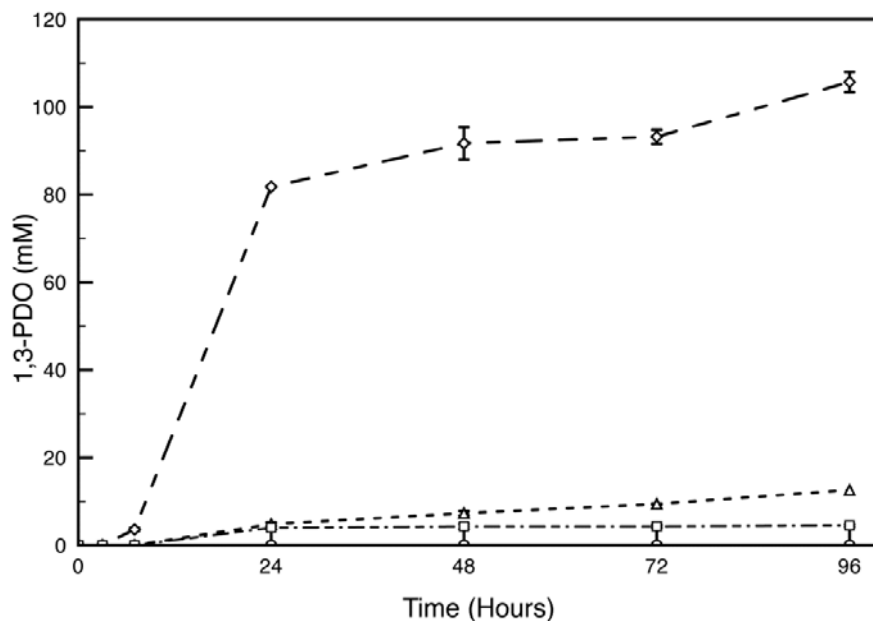


Figure 3.2 The effect of carbon source on 1,3-PDO production
A culture of *L. panis* PM1 was grown in MRS and re-suspended in mMRS containing 200 mM of glycerol and 50 mM of glucose (—◇—), sucrose (—△—), xylose (—○—), and no additional sugar (—□—). The production of 1,3-PDO was determined every 24 hours by proton NMR in comparison to the known molar concentration of an internal DMF standard

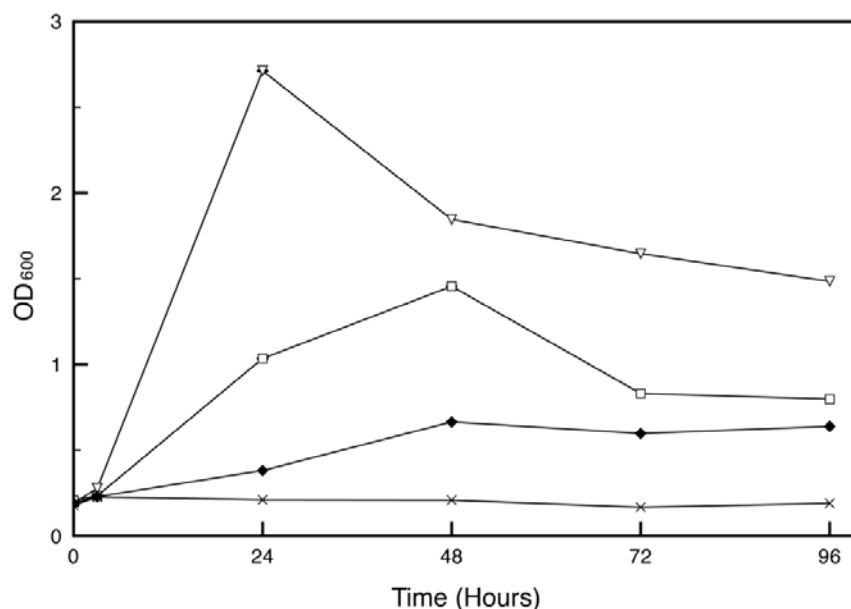


Figure 3.3 The effect of carbon source on the growth of *L. panis* PM1
L. panis PM1 was grown in MRS and re-suspended in mMRS containing 200 mM of glycerol and 50 mM of glucose (—▽—), sucrose (—◆—), xylose (—*—), and no additional sugar (—□—). The production of 1,3-PDO was determined every 24 hours by proton NMR in comparison to the known molar concentration of an internal DMF standard.

Table 3.4 Effects of aeration on the production of 1,3-PDO in a *L. panis* PM1 culture re-suspended in mMRS

Product	Conditions	Observed Accumulation (mM)	Molar Conversion Ratio (%)
1,3-PDO	No Aeration	52.0 \pm 0.1	34.7 \pm 0.1
1,3-PDO	Aeration	24.0 \pm 0.3	16.0 \pm 0.2
Ethanol	No Aeration	13.5 \pm 0.2	9.0 \pm 0.1
Ethanol	Aeration	6.8 \pm 0.1	4.5 \pm 0.1
Acetic Acid	No Aeration	64.8 \pm 0.5	43.2 \pm 0.3
Acetic Acid	Aeration	85.3 \pm 0.2	56.9 \pm 0.1

The resuspended culture of *L. panis* in mMRS culture was split and one sample was agitated at 150 rpm where the other remained static. Both cultures were incubated at 37°C with 50 mM glucose and 100 mM glycerol and were monitored at 0 and 96 hours for changes in media composition. Changes in the concentration of ethanol, acetic acid, and 1,3-PD were monitored by proton NMR.

3.4.4 Effects of initial glycerol concentration on 1,3-PDO production

The highest concentrations of 1,3-PDO were produced when glycerol was kept in the range of 150 – 250 mM. The molar conversion efficiencies were calculated based on the concentrations of glycerol added versus the concentrations of 1,3-PDO produced. For the cultures grown in 25 mM and 1 M glycerol, representing the lowest and highest concentrations of glycerol added an average 1,3-PDO concentration of 21.1 ± 0.1 mM and 8.0 ± 0.1 mM was produced, respectively. The conversion efficiency at these glycerol concentrations were calculated as 84.6 ± 0.4 and 0.8 ± 0.1 % for the 25 mM and 1 M cultures, respectively. Overall the data demonstrated the trend of high conversion efficiencies at the lowest glycerol concentrations (Figure 3.4). The data indicates that concentrations of glycerol from 150 – 200 mM are the most capable in terms of 1,3-PDO production and efficiency. In this range, this experiment showed the conversion rates were 30 – 50% and the concentrations of 1,3-PDO were 70 to 80 mM. Additionally, the growth of the organism was monitored over the trial period and it was found that those in both the lowest and highest concentrations of glycerol did not reach an equivalent OD_{600} as samples containing glycerol in the 50 mM to 200 mM range. Samples with 50 to 200 mM of glycerol had an average final OD_{600} of 2.52 ± 0.04 . For samples ranging from 400 mM to 1 M glycerol the average final OD_{600} was 2.17 ± 0.22 with the 1 M sample showing a 15.7 ± 0.1 % decrease in growth from the average maximum. Samples with no glycerol added showed a maximum OD_{600} of 2.04 ± 0.01 .

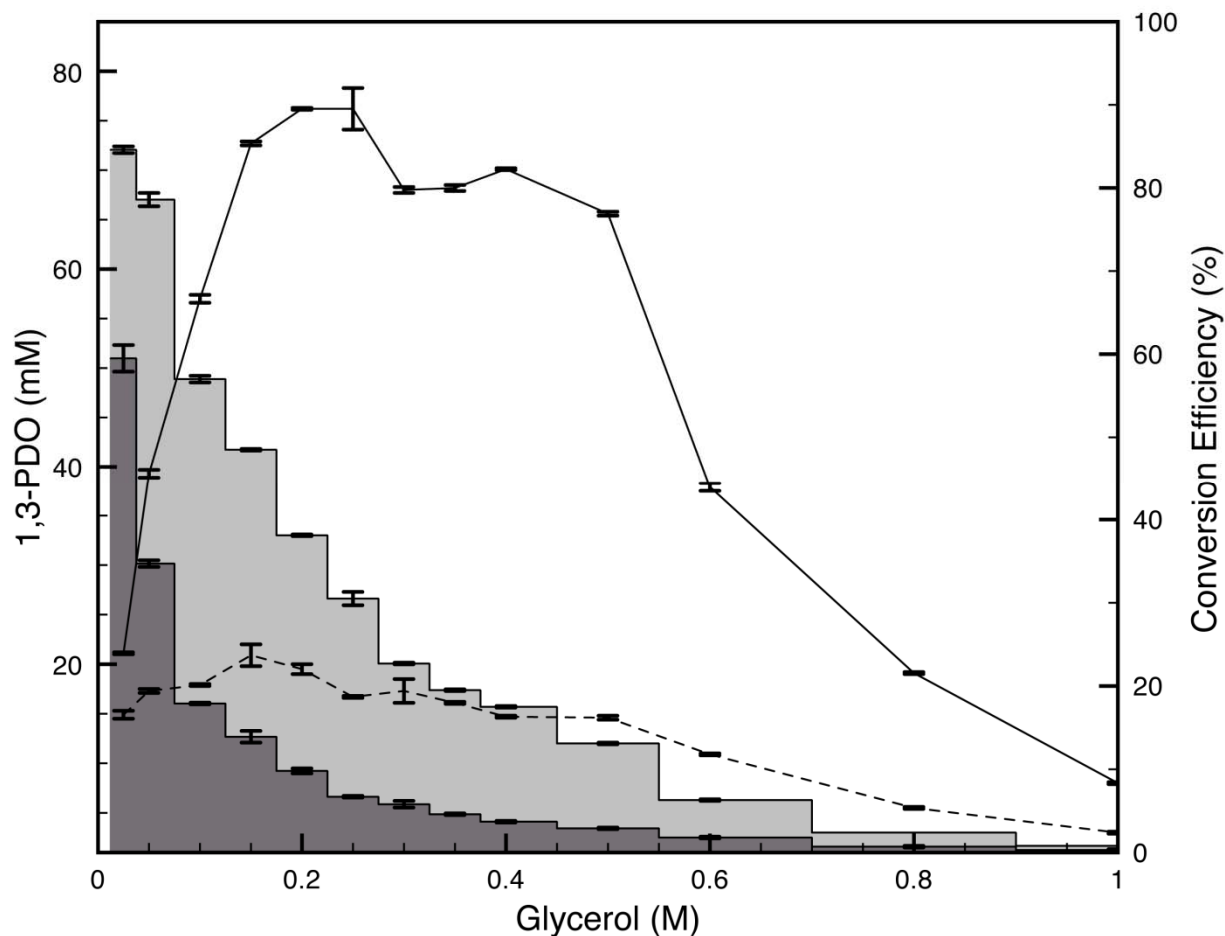


Figure 3.4 The effect of glycerol concentration on the production of 1,3-PDO
A culture of *L. panis* PM1 was grown in MRS and re-suspended in mMRS containing 50 mM glucose and glycerol ranging from 25 mM to 1 M at a pH of 6.2. The concentration of 1,3-PDO was determined at 24 hours (— · —) and 48 hours (— — —). The concentration of 1,3-PDO produced was divided by the concentration of glycerol added to yield the conversion efficiency at 24 hours (Dark grey) and 48 hours (Light grey)

3.4.5 Effects of initial pH on 1,3-PDO production

The quantities of 1,3-PDO produced from pH 5.5 to pH 8 fall within the range of 60 – 90 mM as seen in previous experiments utilizing similar concentrations of glycerol to this experiment (Figure 3.5). However, pH 9 and pH 10 show a far greater level of production achieving final concentrations of 1,3-PDO of 170 ± 4 mM and 222 ± 2 mM respectively.

Data collected for the pH experiments shows upwards of $75 \pm 0.8\%$ conversion efficiency in the pH 10 samples. Additionally, the change in pH was seen to alter the growth of *L. panis*. As shown in Figure 3.6, a higher initial pH appears to slow the growth of the organism. The final pH

values for all samples fell in the similar values (averaged at a pH 4.11 ± 0.07) after 96 hours of incubation. Higher levels of acetic and lactic acid were observed in the pH 9 and 10 samples as compared to samples at a pH between 5.5 and 8. At a pH of 10 the production of acetic acid reached 213.6 ± 2.1 mM.

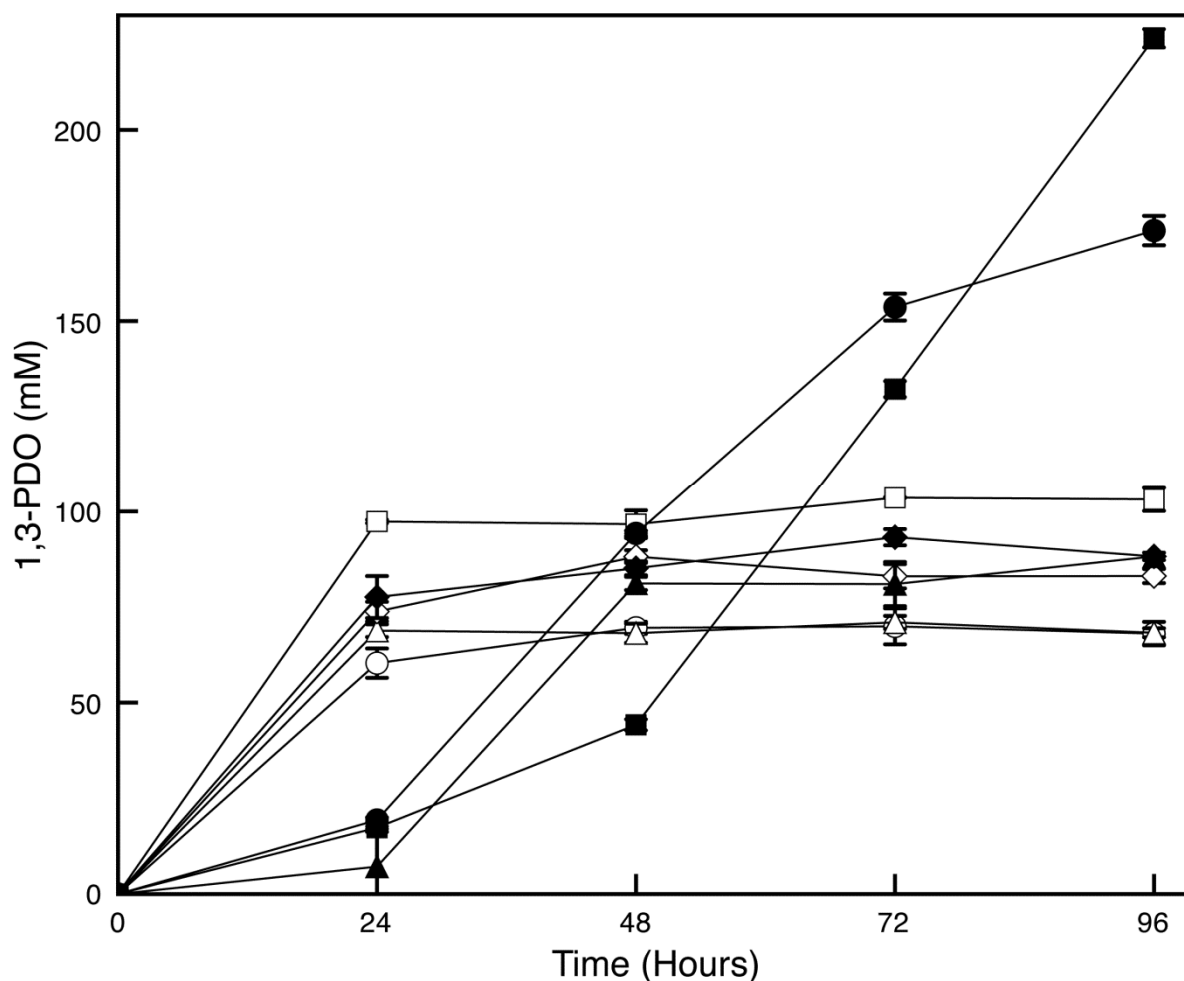


Figure 3.5 The effect of pH on 1,3-PDO production in *L. panis* PM1

A culture of *L. panis* PM1 was grown in MRS and re-suspended in mMRS containing 100 mM glucose and 300 mM glycerol. The concentration of 1,3-PDO was determined every 24 hours for 96 hours. The mMRS media was adjusted such that the initial pH was; pH 5.5 (—□—), pH 6 (—○—), pH 6.5 (—△—), pH 7 (—◇—), pH 7.5 (—▽—), pH 8 (—◊—), pH 9 (—●—), pH 10 (—■—). The production of 1,3-PDO was determined by proton NMR utilizing an internal standard of known molar concentration.

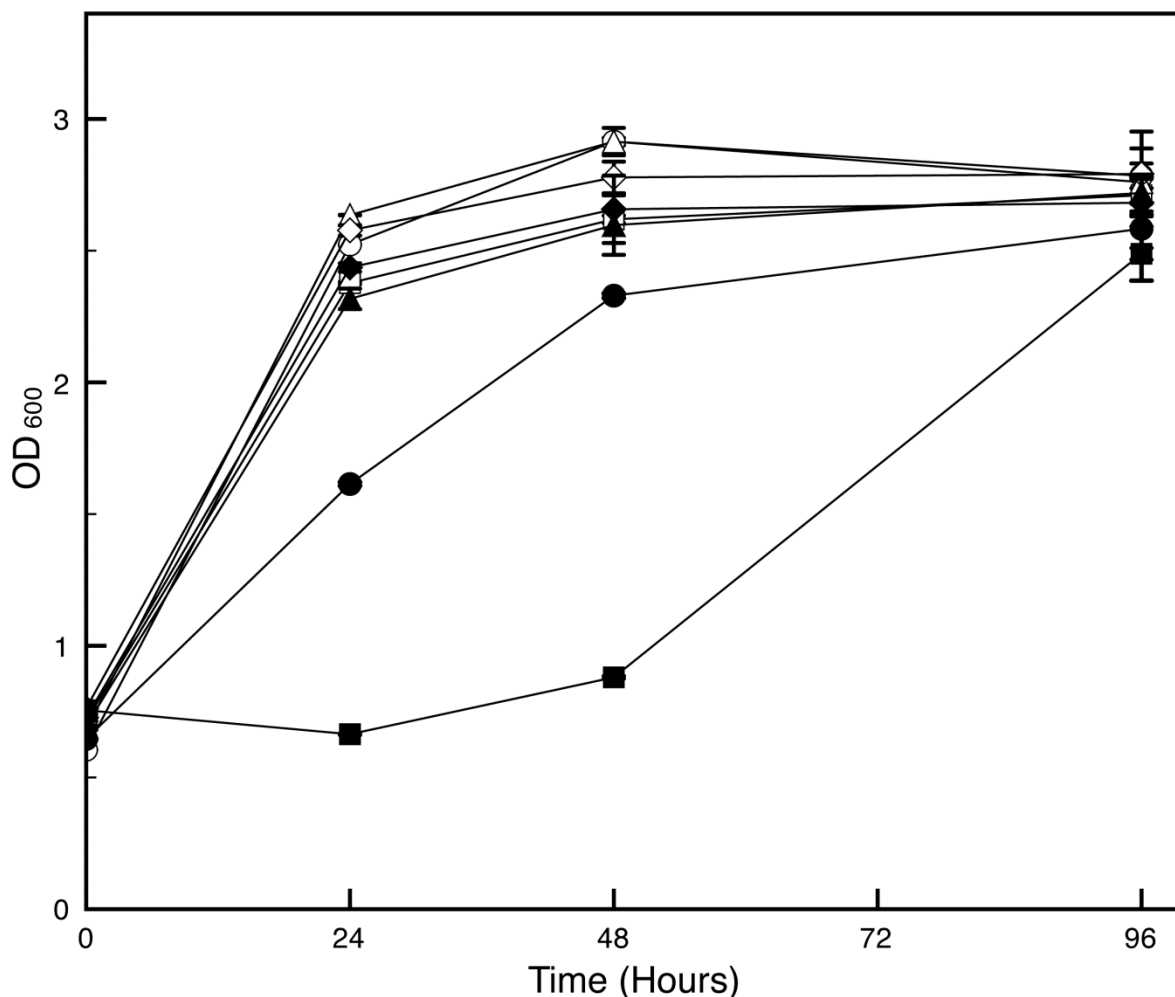


Figure 3.6 The effect of pH on the growth of *L. panis* PM1 re-suspended in mMRS media. A culture of *L. panis* PM1 was grown in MRS and re-suspended in mMRS containing 100 mM glucose and 300 mM glycerol. Cellular growth was determined every 24 hours by observing changes in the OD₆₀₀. The mMRS media was adjusted such that the initial pH was; pH 5.5 (—■—), pH 6 (— —), pH 6.5 (—◊—), pH 7 (—▽—), pH 7.5 (—◊—), pH 8 (—Δ—), pH 9 (—o—), pH 10 (—□—).

3.4.6 Effects of temperature on 1,3-PDO production

The concentrations of 1,3-PDO produced for cultures grown at various temperatures at the 96 hour point are averaged 74.3 ± 2.6 mM with the exception of a possible outlier in the 30°C sample. The 30°C sample produced 99.7 ± 12.1 mM of 1,3-PDO. Given the drastic change in production for the 30°C sample, a Dixon's Q-test was applied to the data. The 30°C sample was rejected as an outlier with 99% confidence. Further analysis of the data demonstrates no significant difference in the final concentration of 1,3-PDO produced at various temperatures.

This lack of variation can be seen as the standard deviation of the final concentration of 1,3-PDO produced. Concentrations of 1,3-PDO produced showed little difference among the 24 samples incubated at 25° - 37°C producing an average of 75.6 ± 1.7 mM. Cultures incubated at 20° and 15°C produced slightly less 1,3-PDO at 67.4 ± 0.5 and 55.9 ± 0.7 mM respectively at 24 hours post inoculation. Data collected indicates that temperature does not play a vital role in the conversion efficiency of glycerol.

3.4.7 Effects of initial cell density on 1,3-PDO production

It was determined that a majority of the 1,3-PDO produced was achieved within the first 24 hours for all of the cultures grown at various initial OD values. The production of 1,3-PDO in the first 24 hours for all samples averaged 43.9 ± 3.0 mM. The final concentration of 1,3-PDO produced at 96 hours for all samples was averaged 50.6 ± 6.3 mM. The data was tested for outliers by a Dixon's Q test. The 72 hour data point for the culture with an initial OD₆₀₀ of 2 was rejected as an outlier with 95% confidence. The standard deviation for the final concentration of 1,3-PD for all samples, excluding outliers, was ± 2.4 mM. The results indicate that the initial cell density had little influence in glycerol conversion.

3.5 Discussion

Our previous study (Khan et al. 2012) indicated the secondary metabolic nature of 1,3-PDO production. It suggested to us the necessity of a method capable of ensuring similar secondary metabolism among samples in order to examine the optimum conditions for the production of 1,3-PDO. As the growth profile and nutrient utilization may fluctuate among cultures it becomes vital that unintentional culture variations do not overshadow the effects of the examined factors. In order to ensure the consistent production of secondary metabolites a culture method utilized by Luthi-Peng et al. was adopted for our *L. panis* PM1 isolate (Luthi-Peng et al. 2002). This method provides a means of simulating a stationary phase culture in a defined media, without the influences of culture lot variations, by resuspending starving, stationary-phase cells in a defined low-sugar media. Consistent and reproducible results were ensured as the culture method starves the cells and depletes the nutrients available to the culture, thus the influences of various culture conditions on both primary and secondary metabolism should be observed. Having selected a culture method capable of consistently, and reproducibly producing conditions for the production of secondary metabolites, the effects of various process parameters were examined.

Initial tests employing Luthi-Pengs' method (Figure 3.1; Table 3.1) indicate that the 1,3-PDO production by this *L. panis* PM1 isolate is associated with ethanol production and glucose consumption. The production of 1,3-PDO does not occur until glucose is depleted to a certain concentration and cellular growth reaches a plateau due to the depletion of glucose. It clearly indicates the secondary metabolic nature of 1,3-PDO production and the method employed is appropriate to examine the effects of culture parameters. Also, Table 3.2 indicates that aeration is a critical factor to change the direction of metabolism into 1,3-PDO production.

The fermentation of glycerol can generate 1,3-PDO as well as aid in the generation of biomass and the production of energy products for Clostridia and Enterobacteriaceae (Willke and Vorlop 2008; Tobajas et al. 2009). The accumulation of biomass and energy production occurs through the reduction of glycerol to dihydroxyacetone (DHA) or glycerol-3-phosphate (G3P) which can then both be reduced to dihydroxyacetone phosphate (DHAP) and ultimately Acetyl-CoA. However, reports have indicated that for other 1,3-PDO producers only a small

percentage of the glycerol available is put towards increasing biomass or the production of energy products (Streekstra et al. 1987; Chen et al. 2003). Data obtained in previous studies have indicated the inability of the PM1 isolate to survive solely on glycerol and thus we concluded that the PM1 isolate is similar to other 1,3-PDO producers in its ability to only utilize a small percentage of the glycerol available is put towards increasing biomass or the production of energy products (Khan et al. 2012). It has been previously demonstrated that the expression of the pathway responsible for the conversion of glycerol to G3P or DHA then to DHAP are, in part, regulated by the presence of oxygen and the intracellular redox-balance (Pasteris and Strasser De Saad 2009). Therefore, increased concentrations of oxygen would promote the reduction of glycerol to G3P and DHAP and its subsequent reduction through the Embden-Meyerhoff-Parnas pathway. The presence of oxygen would also ensure the recycling of NADH through an NADH oxidase given the superior reducing potential of oxygen. An examination of the effects of aeration on the production of 1,3-PDO by *K. pneumoniae* by Wang et al. have shown that completely anaerobic conditions negatively affected the production of 1,3-PDO (Wang et al. 2011). Additionally, Doleyres et al. determined that anaerobic conditions in *Lactobacillus reuteri* lead to increased levels of 3-HPA in a study utilizing a similar two step culture method (Doleyres et al. 2005). These results indicate that oxygen plays an important role in directing the pathways associated with the metabolism of glycerol to 1,3-PDO.

Our previous study (Khan et al. 2012) detailing the inability of the bacterial isolate to survive solely on glycerol indicates the possibility that the bacterial isolate lacks the enzymes responsible for the conversion of glycerol to G3P and/or DHAP. Also, the production of 1,3-PDO in the PM1 isolate is greatly reduced in the presence of oxygen (Table 3.2). Ultimately, the relation among metabolism shunting, oxygen availability, and 1,3-PDO production must be examined to confirm the metabolic activity in the bacterial isolate. As a result of the inability of *L. panis* PM1 to survive solely on glycerol, an alternative energy source is required in low carbon conditions. Given the various energy resources required not only for the production of 1,3-PDO, but for the survival of the organism, the choice of carbon source was examined to determine its effect on 1,3-PDO production. We have identified a small set of possible carbon sources capable

of being metabolized and therefore producing sufficient energy for the growth, survival, and production of 1,3-PDO.

The present study illustrates the importance of glucose in the production of 1,3-PDO as only glucose was capable of producing significant quantities of 1,3-PDO. It was determined that sucrose was able to act as a sufficient carbon source for limited growth of the organism. The ability of glucose to provide an adequate supply of energy metabolites in the production of 1,3-PDO is amplified when compared to the samples grown without glucose. Although the culture with no additional carbon source had sufficient resources to grow adequately it was unable to produce 1,3-PDO. If we look at the resuspended samples in sucrose and xylose in combination with the sample that did not contain an additional carbon source, the production of 1,3-PDO is extremely low. As a result it has been determined that sucrose and xylose appear unable to act as an adequate carbon source whereas glucose appears to be required for the production of 1,3-PDO.

In an effort to extend the period of 1,3-PDO production, sub-optimal temperatures were examined for changes in 1,3-PDO production. The optimal temperature for growth was defined as 37°C (Khan et al. 2012). The data collected from these experiments indicates that temperature did not appear to significantly affect 1,3-PDO production. The data collected appears to reflect this phenomenon as the final concentrations of 1,3-PDO for the various temperatures only deviated by ± 2.6 mM. The lack of variation in the final concentration of 1,3-PDO indicates that extenuating factors, not temperature, played a vital role in determining the final concentration of 1,3-PDO. The lack of a significant difference may indicate that the two-step fermentation method masked the effect of temperature on production as the initial cultures were cultivated at 37°C. As such, temperature should not be ruled out as a means of increasing 1,3-PDO production.

To determine the effect of cell density on 1,3-PDO production, cultures were prepared to give an absorption at an OD₆₀₀ to 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2. The results supported the conclusion that cell density did not play a role in 1,3-PDO production. Given the consistency seen in the average yield of 1,3-PDO, the data may support previously published data indicating that the available glucose (therefore NAD⁺/NADH pool) and the accumulation of 3-HPA are the

major limiting factors in the production of 1,3-PDO, not cell density (Barbirato et al. 1996; Luthi-Peng et al. 2002; Zheng et al. 2011). Statistically insignificant differences were seen in the levels of 1,3-PDO produced at various cellular densities. Therefore, the effect of the two-step fermentation method in masking the effect of cell density on 1,3-PDO production should be further explored.

To determine the optimal concentrations of glycerol for the production of 1,3-PDO, various concentrations of glycerol were tested. Optimal concentrations of glycerol will be defined as those that produce the highest concentration of 1,3-PDO most efficiently without negatively impacting biomass accumulation. Previous reports by Tobajas et al. examining the effect of glycerol concentration on *L. reuteri* found that a glycerol concentration higher than 100 mM was necessary for the production of 1,3-PDO (Tobajas et al. 2009). Further, it was found that for *L. reuteri* the concentration of glycerol did not appear to affect biomass growth (Tobajas et al. 2009). In our study, we determined that 1,3-PDO production occurred at concentrations of glycerol as low as 25 mM to as high as 1 M with the highest levels of combined production and conversion efficiencies for 1,3-PDO occurring over a 150 – 250 mM range. This optimal range of glycerol concentrations is equates to a glucose to glycerol ratio of 0.2 to 0.33. In *L. reuteri*, optimal production of 1,3-PDO occurred at ratios of glucose to glycerol of 0.05, 0.06, and 0.08 (Vollenweider and Lacroix 2004; Tobajas et al. 2009). This indicates that the metabolic utilization of glucose in *L. panis* PM1 differs from *L. reuteri* in the utilization of glucose in the fermentation of glycerol.

Previous studies done by Vanajakshi and Annapurna on a *K. pneumoniae* indicate that pH may be one of the most important process parameter in 1,3-PDO production (Vanajakshi and Annapurna 2011). Samples for the tested pH values were capable of producing 1,3-PDO and able to decrease the pH to an average of 4.11 ± 0.07 at the 96-hour mark. Of interest is the delay in growth and increase in 1,3-PDO production seen in the pH 9 and pH 10 samples. The pH 9 and pH 10 samples indicate that a higher pH delays growth and possibly hinders survival of the organism past a pH of 10. However, the hostile environment produces 1,3-PDO to a higher final concentration, albeit at a slower initial rate. Studies involving salt stress and its associated effect on 1,3-PDO production indicate that the increased salt concentration would not have played a

role in 1,3-PDO production (Xu et al. 2008). Additionally *L. panis* PM1 tolerates up to 12% NaCl concentration (Khan et al., 2012). It is unlikely 1,3-PDO production was affected by the salt concentration changes associated with pH adjustment. Therefore the elevated pH appears to be primarily responsible for increase in the production of 1,3-PDO. We speculated that the induction of a basic environment stimulates the production of acetic and lactic acid from the organism to produce a more hospitable environment for survival. The pathways responsible for the production of lactic and acetic acid require NAD^+ and produce NADH along with their end products. To maintain the redox-balance an increase in the production of 1,3-PDO as a means of regenerating NAD^+ occurs. As a result, the production of 1,3-PDO occurs and is extended and increased as a byproduct of the lactic and acetic acid necessary to create a more hospitable environment for the growth and survival of the organism. Additionally, the elevated pH may be sufficient to reduce the activity of any enzymes competing for similar energy resources, thereby, increasing the supply of NAD^+/NADH to the 1,3-PDO pathway.

Without an appropriate supply of ATP, NAD^+ , and NADH, the fermentation of glycerol would not occur. The inability of the samples to produce 1,3-PDO in the absence of glucose indicates that for those samples the NAD^+/NADH pool and various energy metabolites required were not available for 1,3-PDO production. The importance of the NAD^+/NADH pool in the production of 1,3-PDO was highlighted in the ability of the sample containing glucose to produce 1,3-PDO; the supplied glucose was sufficient for the generation of an adequate NAD^+/NADH pool in the production of 1,3-PDO. Generally speaking, the pools of these substances are maintained through consumption of high energy carbon substances, i.e., sugars. However, the 1,3-PDO production in *L. panis* PM1 occurs under carbon limited conditions. Thus, *L. panis* PM1 should supplementarily use an alternative pathway to produce ATP and maintain NADH/NAD^+ balance. It has been previously determined by Veiga Da Cunha and Foster (1992) that the production of 1,3-PDO appears to be involved in the reoxidation of NADH to NAD^+ to allow lactate, among other energy metabolites, to be scavenged to generate additional energy currencies such as ATP in *Lactobacillus brevis* and *L. buchneri*. By converting 1 mol of lactate to acetate, 1 mol of NADH can be conserved. Additionally, by converting 1 mol of pyruvate to acetate instead of lactate, 2 mol of NADH may be conserved and an additional 1 mol of ATP may

be generated (Veiga Da Cunha and Foster 1992). The additional energy scavenged is thought to aid survival and growth of the organism under carbon limited and/or fermentative conditions (Veiga Da Cunha and Foster 1992; Pasteris and Strasser De Saad 2009). However, this redirection of the metabolism will produce extra NADH; therefore, the redox potential must be retained through another conversion of NADH to NAD^+ . The present study suggested that the production of 1,3-PDO will play an important role in maintaining the redox-balance of the organism by recycling the accumulated NADH produced during the shift in metabolism to produce acetate at low carbon concentrations. This speculation is in accordance with higher initial pH conditions becoming reduced by higher accumulated levels of acetate and lactate associated with higher production levels of 1,3-PDO by *L. panis*.

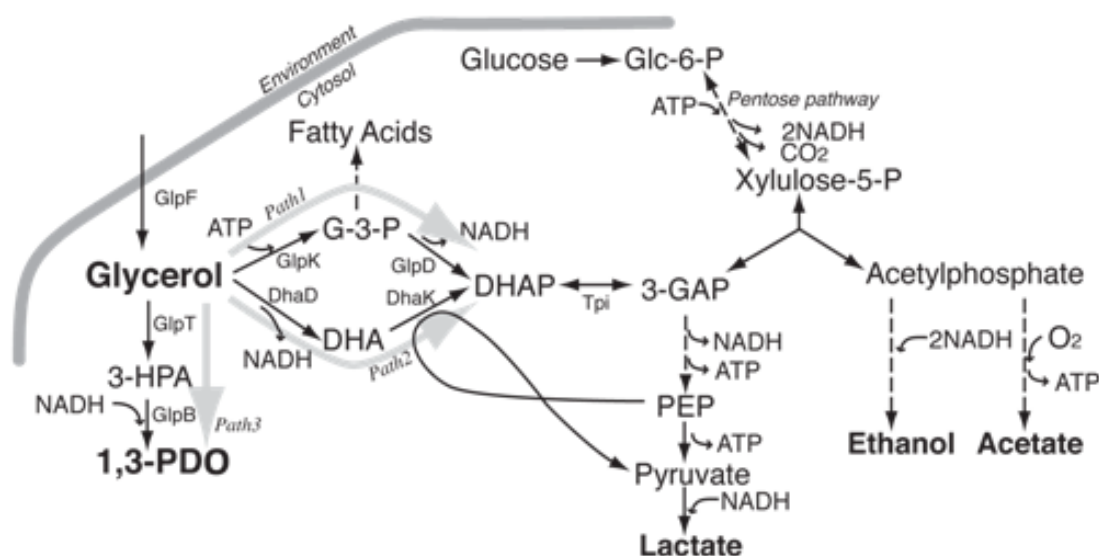


Figure 3.7 - Proposed pathway for the conversion of glycerol to 1,3-PDO and the role of glycerol in anaerobic metabolism.

Enzymes involved in the metabolism of glycerol

Path 1: GlpK - Glycerol kinase, GlpD - Glycerol-3-phosphate dehydrogenase

Path 2: DhaD - Glycerol 2-dehydrogenase DhaK - Dihydroxy acetone kinase

Path 3: GlpT - Glycerol dehydratase, GlpB - 1,3-PDO NAD^+ -dependent oxidoreductase

Tpi - Triose-phosphate isomerase

Metabolites/Substrates in the metabolism of glycerol

Path 1: G-3-P - Glycerol-3-phosphate, DHAP - Dihydroxyacetone-phosphate

Path 2: DHA - Dihydroxyacetone

Path 3: 3-HPA - 3-Hydroxypropionaldehyde, 1,3-PDO - 1,3-Propanediol

3.6 Conclusion

In summary, the production of 1,3-PDO is a mechanism to recover and recycle NADH to NAD^+ under fermentative and low carbon conditions in many 1,3-PDO producers (Johnson and Lin 1987; Talarico et al. 1990; Veigadacunha and Foster 1992). The pathways responsible for the fermentation of glycerol and the ultimate production of 1,3-PDO are complex and affected by a variety of environmental and cellular conditions. For our *L. panis* PM1 isolate, optimal conditions for the production of 1,3-PDO from glycerol were identified as a static culture containing 150-250 mM glycerol, 50 mM glucose at an elevated pH 9 – 10 at 37 °C. Further, the utilization of glucose in the fermentation of glycerol has been shown to differ from *L. reuteri* as seen in the ratios of glucose to glycerol required for 1,3-PDO production. The variance in glucose to glycerol ratios between *L. reuteri* and *L. panis* combined with the inability of *L. panis* to survive solely on glycerol indicate differences in the metabolic pathways associated with 1,3-PDO production. The inability of *L. panis* to survive on glycerol solely indicates that the absence of pathways responsible for the generation and utilization of either or both G3P and DHAP from glycerol. Competition for NADH would exist between metabolic pathways responsible for producing energy products and those responsible for maintaining the redox balance of the organism. Therefore, maintaining an appropriate level of NAD^+/NADH may ultimately limit the production of 1,3-PDO in our, and possibly other, microbial producers (Zheng et al. 2011). To answer this, a further understanding and optimization of the metabolic pathways associated with 1,3-PDO production will be necessary and is currently underway in our research group.

3.7 Connection to next study

A number of factors have been shown to effect the production of 1,3-PDO from bacterial isolates (Streekstra et al. 1987; Barbirato et al. 1996; Ahrens et al. 1998; Asad Ur et al. 2008; Xue et al. 2010; Baeza-Jimenez et al. 2011; Wang et al. 2011). A number of these factors limit the capacity of the microorganism to produce industrially relevant quantities of 1,3-PDO. Given the industrial interest in the microbial production of 1,3-PDO strategies to circumvent the associated issues related to the production of industrially relevant quantities of 1,3-PDO must be developed. Fed-batch and continuous cultures have proven capable of producing significant and continuous quantities of industrially relevant products (Ito et al. 1991; Menzel et al. 1997; Xue et al. 2010). Utilizing the data collected from Study 1 a preliminary examination of a fed-batch model of production by *L. panis* PM1 was undertaken in an attempt to increase both the quantity and duration of 1,3-PDO production.

4. STUDY 2 - FED-BATCH PRODUCTION OF 1,3-PDO FROM *L. PANIS* PM1

4.1 Abstract

The utilization of microorganisms is currently being explored as a means of producing traditionally chemically synthesized products in a more environmentally cost-effective manner. In particular, research into the microbial production of 1,3-propanediol (1,3-PDO) has garnered a large amount of interest from the petrochemical industry. The ability of a novel *L. panis* PM1 isolate to produce industrially relevant levels of 1,3-PDO in a fed-batch culture was explored. Culture utilizing a feed and batch culture at an initial pH of 8, 9, and 10 supplemented with glucose and glycerol was explored. It was determined that a batch culture at an initial pH of 8 containing 50 mM glucose and 150 mM glycerol fed with a broth at a pH of 8 with 5 mM glucose and 150 mM glycerol produced the highest concentrations of 1,3-PDO. Optimized conditions produced a final concentration of 85.3 ± 1.0 mM of 1,3-PDO over 120 hours. The duration and mole concentration of 1,3-PDO produced were improved in comparison to that of a static culture. However, the molar concentrations of 1,3-PDO produced were found to be similar to that of a static culture.

4.2 Introduction

The ability to produce a variety of desirable chemical products from a single common source has led to an increased interest in the linear aliphatic compound 1,3-PDO (Van Haveren et al. 2008). This interest stems from the ability of 1,3-PDO to act as a platform chemical in the generation of polyester and plastic based materials (Biebl 1999; Xue et al. 2010). The two primary chemical methods for the production of 1,3-PDO are known as the Shell or Degussa processes, respectively (Kraus 2008).

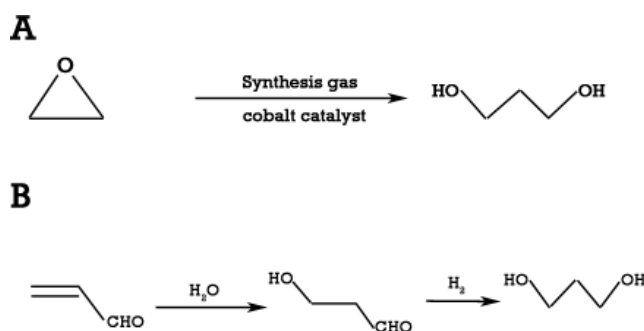


Figure 4.1 Hydroformylation of ethylene oxide
(A) Shell method of 1,3-PDO production (B) Degussa conversion of acrolein to 1,3-PDO

Unfortunately, the production of 1,3-PDO through traditional chemical synthesis can be inefficient and lead to the formation of toxic byproducts (Vollenweider and Lacroix 2004; Mu 2006). The rising cost of crude oil required for the chemical synthesis of 1,3-PDO, plastics, and polyesters have begun to erode the profit margins of traditional chemical synthesis. Increasing costs and ecological concerns have stimulated research aimed at improving the yield, productivity, while reducing the economic concerns associated with microbial production of 1,3-PDO (Gungor and Gupta 1999; Willke and Vorlop 2008). Efforts to improve yields and the productivity of bacterially-produced 1,3-PDO has led to research involving the optimization of process parameters, genetic engineering, as well as various fermentation strategies (Silva et al. 2009; Xue et al. 2010).

The use of a continuous culture for the production of 1,3-PDO has shown great promise with rates of 1,3-PDO production ranging from 64.4 to 115.7 mmol/L/h (Xue et al. 2010). However, continuous cultures have shown an overall reduced yield of 1,3-PDO with final

concentrations reaching a maximum of 0.64 mol/L (Xue et al. 2010). In comparison to continuous cultures, fed-batch cultures have been shown to produce 1,3-PDO to levels as high as 0.92 mol/L, albeit at a slower rate (Xue et al. 2010). Utilizing previously published data on the optimized process parameters for the production of 1,3-PDO from *Lactobacillus panis* PM1, a fed-batch culture was employed in an effort to both increase and produce a continuous stream of 1,3-PDO (Grahame et al. 2012).

Objectives of this study were:

- A) To determine the ability of the *L. panis* PM1 culture to produce 1,3-PDO in a fed-batch culture;
- B) To increase the duration and mole quantity of 1,3-PDO production; and
- C) To gain additional insight into the metabolic control over 1,3-PDO production in *L. panis* PM1.

4.3 Materials and Methods:

4.3.1 Analytical Determination and Quantification of Cellular Growth, 1,3-PDO and Major Metabolite Concentrations

Cellular growth was determined by observing the changes in the absorbance at an optical density of 600 nm (OD_{600}) in a Beckman DU800 spectrophotometer. Samples were performed in triplicate and for samples with an OD_{600} reading over 1.00, a one in ten dilution was made in H_2O to ensure a more accurate OD reading.

Concentrations of glucose and glycerol were determined by the Free Glycerol Determination Kit (Sigma Chemicals, St Louis, MO, USA; cat# FG0100) and Glucose (HK) Assay kit (Sigma Chemicals, St Louis, MO, USA; cat# GAHK-20) following the manufacturers guidelines. Samples for these assays were prepared using centrifugation at $12,000 \times g$ in a bench top centrifuge and the supernatant was passed through a $0.45\text{-}\mu\text{M}$ filter to remove any remaining bacterial cells and large particulate materials.

Concentrations of 1,3-PDO and other major metabolites, e.g. ethanol, lactic acid, and acetic acid, were determined by proton nuclear magnetic resonance as above (NMR Model AMX 500-MHz, NMR Bruker, Mississauga, ON) at the Saskatoon Structural Sciences Center (SSSC University of Saskatchewan).

4.3.2 Fed-Batch production of 1,3-propanediol

The growth of *L. panis* PM1 was conducted as follows unless otherwise stated; *L. panis* PM1 was grown in 50 mL of MRS (DIFCO, Sparks, MD, USA) broth in a 50-mL conical tube at 37°C for 24 hours. The *L. panis* culture was cultured under static conditions with no oxygen introduced to the culture through agitation. Cells were harvested by centrifugation at $11,200 \times g$ in an SS-34 rotor in a Sorval high-speed centrifuge at 4°C for 20 minutes. The cell pellet was re-suspended and washed with deionized H_2O twice. The washed cell mass was re-suspended in one tenth of the original volume in a MRS broth lacking acetate and Tween 80 at a defined pH, hereafter referred to as modified MRS (mMRS), containing 50 mM glucose and 150 mM glycerol. The mMRS media was composed of 5 g yeast extracts, 10 g tryptone, 5 g meat extracts,

2 g K_2HPO_4 , 2 g ammonium citrate, 5 g sodium acetate, 100 mg $MgSO_4 \cdot 7H_2O$, 50 mg $MnSO_4$, per liter. A 15-mL aliquot of re-suspended culture was added to a sterile 135-mL mMRS broth containing 50 mM glucose and 150 mM glycerol in a 500-mL baffled flask hereafter referred to as the batch culture. The batch culture was grown at 37°C under static conditions in micro-anaerobic conditions for 12 or 24 hours. After the 12 or 24 hour incubation period the feed broth, composed of sterile mMRS broth, containing a defined concentration of glycerol and glucose, was added at a rate of 2.3 mL per hour by a Marlow-Watson 200 (Massachusetts, USA) peristaltic pump. The feed broth contained 150 mM glycerol and 5 mM or 10 mM of glucose where the pH was altered by the addition of 1 M HCl or 1 M NaOH depending upon the trial. A 1-mL sample was taken every 24 hours for proton NMR analysis to determine metabolite concentrations. The growth and pH of the fed-batch culture was monitored by a Beckman Coulter DU800 spectrophotometer and a VWR Symphony SB70p pH meter, respectively.

4.4 Results

4.4.1 Fed-Batch production of 1,3-propanediol

Initial fed-batch trials utilized a pH of 10 and 9 for both the batch culture and feed broth. Addition of the mMRS feed broth began 24 hours after inoculation and continued for a period of 96 hours. Titrers of 1,3-PDO were recorded over 120 hours and the final titer of 1,3-PDO produced can be seen in Table 4.1.

Table 4.1 Production of 1,3-PDO in initial fed-batch pH 10 and 9 trials

Chemical	Trials	1,3-PDO (mM)
1,3-PDO	pH 10 - 120 Hours	8.1
1,3-PDO	pH 9 - 120 Hours	13.3

As initial trials were incapable of producing significant quantities of 1,3-PDO at a pH of 9 and 10 additional trials were undertaken with the batch and feed broth adjusted to a pH of 8. An increase in 1,3-PDO production was seen when culture conditions were shifted to a pH of 8 (Figures 4.2 and 4.3). Subsequently, additional trials were performed utilizing a pH 8 batch culture with the following amendments; the feed stock was altered to contain 5 and 10 mM glucose and 150 mM glycerol at a pH of 8 and 9. Additionally, a trial using 50 mM glucose, 150 mM glycerol, and at a pH of 7 for the batch culture was undertaken with a pH 8, 5 mM glucose, 150 mM glycerol feed stock. A comparison of the various culture conditions ability to produce 1,3-PDO can be seen in Figures 4.2 and 4.3.

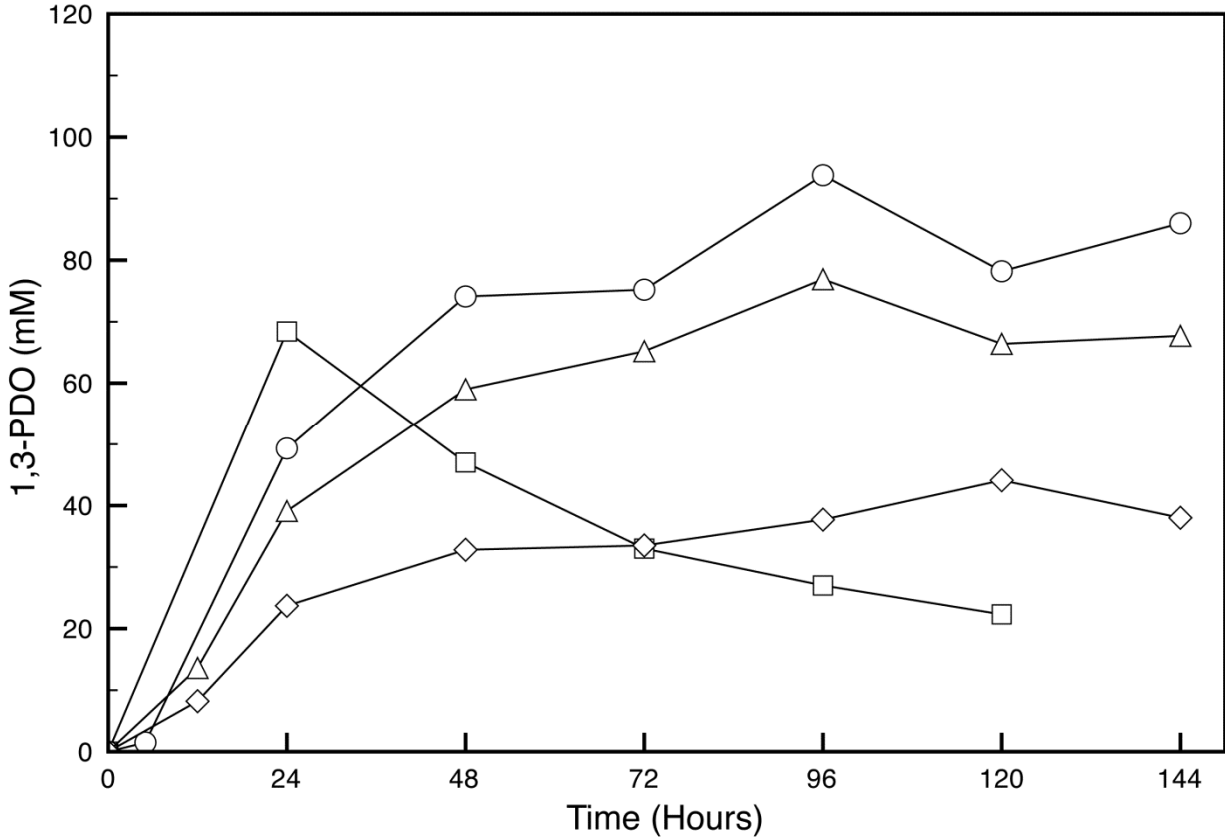


Figure 4.2 Effect of pH and glucose concentration on the fed-batch production of 1,3-PDO by *L. panis* PM1

A culture of *L. panis* PM1 was grown in MRS and re-suspended in mMRS containing 50 mM glucose and 150 mM glycerol adjusted to an initial pH of; pH 7 (—□—), pH 8 (—○—), pH 9 (—△—), and a pH of 10 (—◇—) for the batch culture. The batch culture was fed with a mMRS broth at the following conditions; 5 mM glucose (pH 7) at 24 hours post-inoculation (—□—), 5 mM glucose (pH 8) at 24 hours post inoculation (—○—), 10 mM glucose (pH 8) at 12 hours post-inoculation (—△—), 10 mM glucose (pH 9) at 24 hours post-inoculation (—◇—)

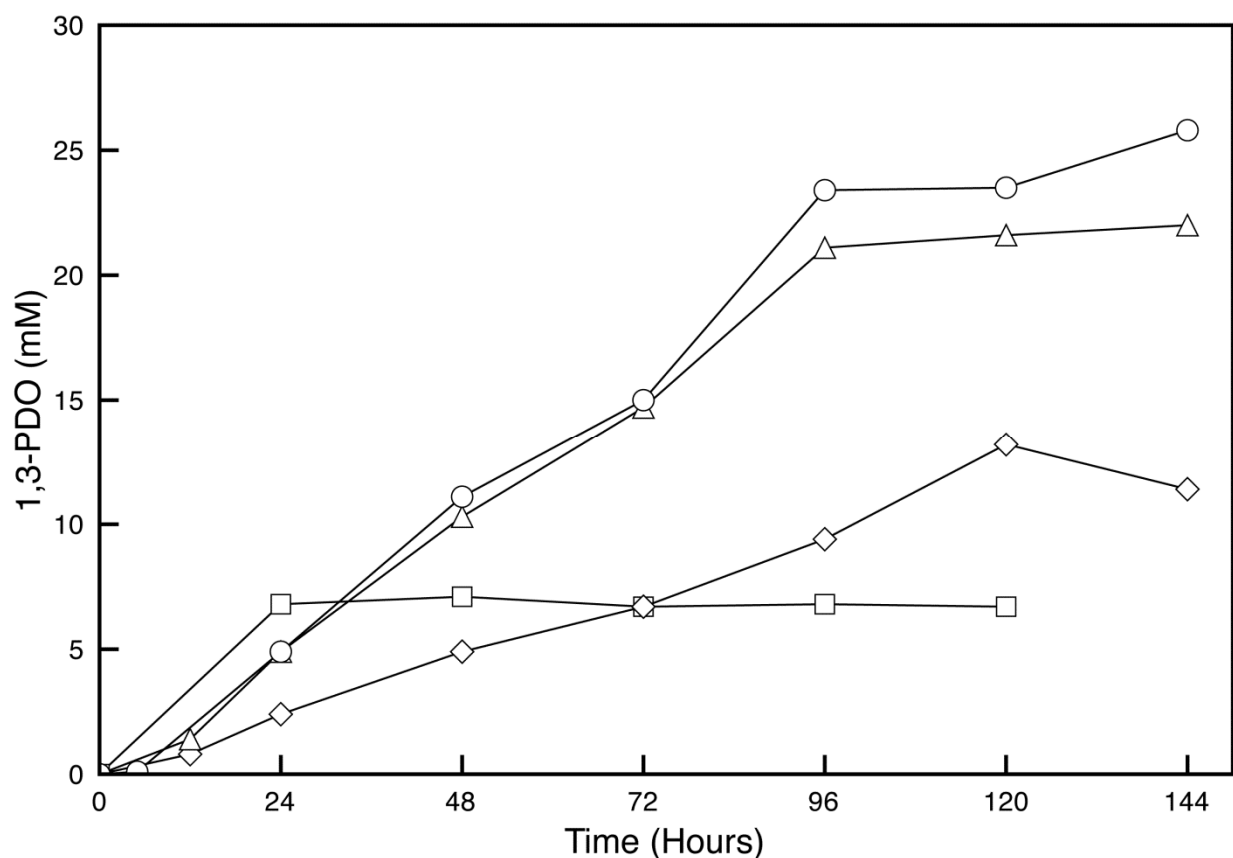


Figure 4.3 Effect of pH and glucose concentration on the fed-batch production of 1,3-PDO by *L. panis* PM1

A culture of *L. panis* PM1 was grown in MRS and re-suspended in mMRS containing 50 mM glucose and 150 mM glycerol adjusted to an initial pH of; pH 7 (—□—), pH 8 (—○—), pH 9 (—△—), and a pH of 10 (—◇—) for the batch culture. The batch culture was fed with a mMRS broth at the following conditions; 5 mM glucose (pH 7) at 24 hours post-inoculation (—□—), 5 mM glucose (pH 8) at 24 hours post inoculation (—○—), 10 mM glucose (pH 8) at 12 hours post-inoculation (—△—), 10 mM glucose (pH 9) at 24 hours post-inoculation (—◇—).

The linear rate of production was determined for the fed-batch culture that produced the highest concentration of 1,3-PDO. The fed-batch culture with the highest concentration of 1,3-PDO was the sample grown in a batch culture at a pH of 8 and supplemented with a pH 8, 5 mM glucose feed stock. The final titer of 1,3-PDO for the pH 8 culture was determined to be 85.3 ± 1.0 mM equating to 29.8 ± 0.03 mmol 1,3-PDO in the 350 mL final volume. The rate of production was determined to be 0.3 mmol of 1,3-PDO per hour with an R^2 value of 0.987. This production rate equates to 23 mg 1,3-PDO per hour.

As per the materials and methods, the growth and pH of the cultures were monitored every 24 hours. A cyclic pattern of growth was observed for all fed-batch trials. The cultures fed with higher concentrations of glucose displayed higher cell densities over time. Changes in growth can be seen in Figure 4.4.

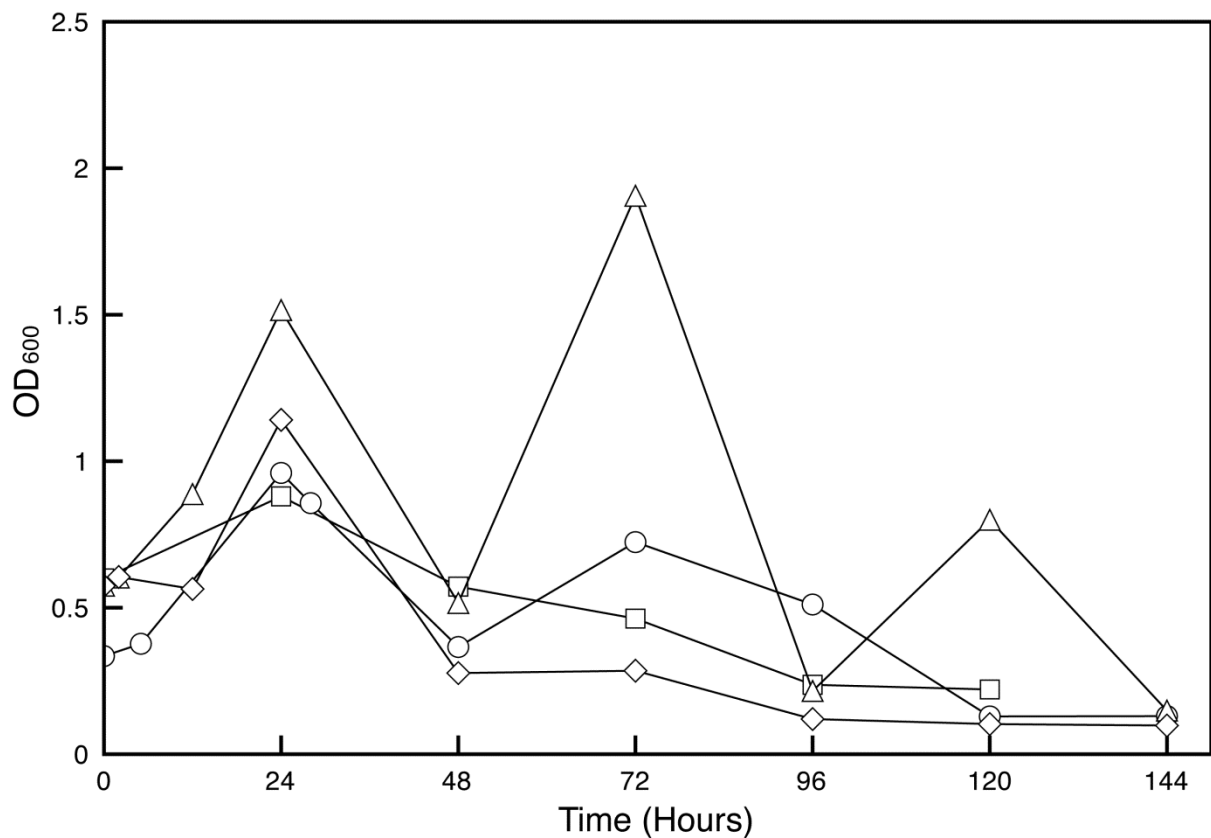


Figure 4.4 Effect of pH and glucose concentration on the growth of a fed-batch culture of *L. panis* PM1

A culture of *L. panis* PM1 was grown in MRS and re-suspended in mMRS containing 50 mM glucose and 150 mM glycerol adjusted to an initial pH of; pH 7 (—□—), pH 8 (—○—), pH 9 (—△—), and a pH of 10 (—◇—) for the batch culture. The batch culture was fed with a mMRS broth at the following conditions; 5 mM glucose (pH 7) at 24 hours post-inoculation (—□—), 5 mM glucose (pH 8) at 24 hours post inoculation (—○—), 10 mM glucose (pH 8) at 12 hours post-inoculation (—△—), 10 mM glucose (pH 9) at 24 hours post-inoculation (—◇—).

4.5 Discussion

Fed-batch and repeated fed-batch methods have proven to be capable of producing significant quantities of a desired end product (Ito et al. 1991; Wan et al. 2005; Seo et al. 2009; Xue et al. 2010). In this study, a fed-batch culture was explored as a means of producing significant quantities of 1,3-PDO from a novel *L. panis* PM1 isolate. Study 1 determined that pH, glucose concentration, and glycerol concentration contributed to the final titer of 1,3-PDO produced in our *L. panis* PM1 isolate. Additionally, Study 1 indicated that an increased pH played a predominant role in increasing the titer of 1,3-PDO produced. Accordingly, a series of fed-batch cultures utilizing the optimized culture parameters for glucose and glycerol defined in Study 1 at various pH levels were explored as a means of further increasing 1,3-PDO titer.

Initial fed-batch cultures were inoculated in an mMRS media with a starting pH of 10 containing 50 mM glucose and 150 mM glycerol. However, as Table 4.1 indicates, the fed-batch culture yielded a small concentration of 1,3-PDO. Given the low levels of 1,3-PDO produced at pH 10, the culture conditions were repeated with the batch culture and feed broth adjusted to a pH of 9 from 10. Table 4.1 illustrates that the change in pH did not have the desired effect as the final titer of 1,3-PDO produced only increased by 5.2 mM. The poor production of 1,3-PDO at a pH of 9 necessitated further changes to the culture conditions leading to a further reduction of the batch and feed mMRS broth to a pH of 8 from a pH of 9. The lack of 1,3-PDO production in combination with the poor growth indicates that the rate and conditions of addition would have led to the biomass being washed out. As such, the cultures at a pH of 9 and 10 would not have been able to produce 1,3-PDO. By altering the pH of the re-suspension and the feed broth to a pH of 8, an increase in 1,3-PDO titer was observed. Concentrations of 1,3-PDO ranged from 86 to 70 mM for fed-batch cultures grown at a pH of 8 (Figures 4.2 and 4.3). The reduction in pH correlates with conditions identified in literature where a controlled pH of 7.0 for a fed-batch and continuous culture of *Klebsiella pneumoniae* produced 66 g/L and 50 – 60 g/L, respectively (Menzel et al. 1997; Xue et al. 2010). The addition of a feed broth or re-suspension broth above or below a pH of 8 reduced the final yield of 1,3-PDO produced (Figures 4.2 and 4.3). A plot of the millimolar concentration of 1,3-PDO produced indicated that cultures grew at a pH of 8 and when supplemented with a feed at a pH of 8 were capable of consistently producing 1,3-PDO

for 96 hours. Utilizing the data for the first 96 hours, the rate of production was determined to be 0.3 mmol, or 23 mg, of 1,3-PDO per hour in a 150-mL culture. A further examination of the data indicated that although 1,3-PDO was produced at a steady rate over the 96 hours, the cell density fluctuated (Figure 4.4). The disparity in cell density between the cultures supplemented with 10 and 5 mM glucose suggests that additional glucose was not directed to the production of 1,3-PDO but rather to cellular growth. Continuous culture and fed-batch experiments utilizing *K. pneumoniae* showed similar initial growth patterns with markedly slower decreases in cell density over time (Menzel et al. 1997; Xue et al. 2010). Supplementation of the feed media with a mixture of organic acids has been shown to aid in 1,3-PDO production and cellular growth (Xue et al. 2010). Given that the additional glucose was utilized for cellular growth, not 1,3-PDO production, the supplementation of the feed media with organic acids, as seen in *K. pneumoniae*, may aid in the survival and growth of the *L. panis* PM1 isolate (Xue et al. 2010). Moreover, it is possible that supplementation of the feed media with alternative carbon sources that decrease competition for the production of 1,3-PDO, or stimulate pathways responsible for it, could increase the final concentration of product produced. With an optimized feed source increased production of 1,3-PDO should be possible, whereas, in our study, limited variation was seen in the quantity of 1,3-PDO produced in the fed-batch cultures containing either 5 or 10 mM of glucose (Figures 4.2, 4.3). The changes in OD₆₀₀ versus the limited variation in 1,3-PDO levels observed would indicate that competition for available energy resources exists between the alternative pathways involved in fermentation (Menzel et al. 1997).

4.6 Conclusion:

The production of 1,3-PDO from a novel *L. panis* isolate were identified in Study 1. One of the primary goals of this work was to increase the levels and duration of 1,3-PDO produced by *L. panis* PM1 through a fed-batch culture. It was determined from the conditions tested that the ability of a fed-batch culture to produce significant quantities of 1,3-PDO from our *L. panis* PM1 isolate depended heavily upon the initial pH of the batch as well as the pH of the feed broth. The highest levels of 1,3-PDO were found to be produced utilizing an initial 50 mM glucose and 150 mM glycerol at a pH of 8 supplemented with a 5 mM glucose 150 mM glycerol feed stock at a pH of 8. Final concentrations of 1,3-PDO were found to be similar to reported levels for the *L. panis* isolate grown in static cultures containing glucose in the range of 5 – 50 mM producing 85.3 ± 11.9 versus 79.7 ± 10.4 mM 1,3-PDO for the fed-batch trial; however, the mole levels of 1,3-PDO produced were significantly higher with 22.3 ± 1.6 versus 5.32 ± 0.7 mmol 1,3-PDO produced for the fed batch versus the static culture. Although an increased quantity and an extended duration of 1,3-PDO production were achieved various factors must be further explored to determine the ability of a fed-batch model to produced industrial relevant concentrations of 1,3-PDO.

4.7 Connection to next study

The potential influence of 3-HPA on 1,3-PDO production has previously been demonstrated (Barbirato et al. 1996; Barbirato et al. 1996; Zheng et al. 2011). The ability of *L. reuteri* to survive and function in the presence of 3-HPA has also been documented (Bauer et al. 2010; Baeza-Jimenez et al. 2011). As a result the NAD⁺-dependent oxidoreductase responsible for converting 3-HPA to 1,3-PDO was cloned in an attempt to generate a recombinant protein capable of mitigating 3-HPA accumulation. Further, given the phylogenetic similarities between *L. reuteri* DSM20016 and *L. panis* PM1, characterization of two enzymes involving in the conversion of glycerol to 1,3-PDO (glycerol dehydratase and NAD⁺-dependent oxidoreductase) may give insight into the optimal conditions and capabilities of our isolate.

5 STUDY 3 - CLONING AND CHARACTERIZATION OF A NAD⁺-DEPENDENT OXIDOREDUCTASE FROM *LACTOBACILLUS REUTERI* DSM 20016

5.1 Abstract

The ability of *L. reuteri* to survive in the presence of, and accumulate large concentrations of 3-HPA is of great interest given the detrimental effects of 3-HPA in other bacteria (Talarico and Dobrogosz 1989; Barbirato et al. 1996; Zheng et al. 2011). Further, the production of 1,3-PDO from *L. reuteri* has been shown to be dependent on the ratio of glucose to glycerol and the NAD⁺/NADH pool (Luthi-Peng et al. 2002). Given the ability of 3-HPA to act as an inhibitory molecule, maintaining an appropriate level of NADH/NAD⁺ is vital to ensuring removal of the toxic intermediate (Barbirato et al. 1996; Barbirato et al. 1996; Doleyres et al. 2005; Bauer et al. 2010; Zheng et al. 2011). Thus, the 1,3-propanediol NAD⁺-dependent oxidoreductase from *L. reuteri* DSM 20016 was cloned and characterized to determine the affinity of the enzyme for 1,3-PDO and NAD⁺/NADH. The V_{\max} value for NAD⁺ was determined to be 0.50 ± 0.19 $\mu\text{mol per minute per mg}$ of crude extract whereas the K_m value for NAD⁺ was determined to be 1.28 ± 0.57 mM. The V_{\max} value for 1,3-PDO was determined to be 0.65 ± 0.07 $\mu\text{mol per minute per mg}$ of crude extract. The K_m value for 1,3-PDO was determined to be 28.4 ± 1.1 mM. The high substrate affinity for NAD⁺/NADH indicates that the availability of NAD⁺/NADH may be a determining factor in 1,3-PDO titer and, therefore, 3-HPA accumulation.

5.2 Introduction

The intermediate 3-HPA has been shown to act as a bactericide, inhibit fermentation, and limit the production of 1,3-PDO in *L. reuteri*, *E. agglomerans*, and other 1,3-PDO producing bacteria (Talarico and Dobrogosz 1989; Barbirato et al. 1996; Barbirato et al. 1996; Vollenweider et al. 2003; Zheng et al. 2011). The inhibitory molecule 3-HPA may be removed from the system by its conversion to 1,3-PDO by an NAD^+ dependent oxidoreductase that consumes NADH. Accordingly, the concentration of NADH in the system is thought to play a role in the final titer of product produced (Barbirato et al. 1996; Luthi-Peng et al. 2002). The NADH consumed by the oxidoreductase is released as NAD^+ aiding in the redox-balance of the organism and recycling the energy intermediate NAD^+ (Johnson and Lin 1987; Barbirato et al. 1996; Barbirato et al. 1996; Gonzalez et al. 2008). Given the ability of 3-HPA to act as an inhibitory molecule maintaining an appropriate level of NADH/ NAD^+ is vital to ensuring removal of the toxic intermediate (Barbirato et al. 1996; Barbirato et al. 1996; Doleyres et al. 2005; Bauer et al. 2010; Zheng et al. 2011). In order to understand the requirement of the NADH/ NAD^+ pool, the enzyme responsible this process should be investigated. Therefore, the oxidoreductase from *L. reuteri* DSM 20016 was cloned, expressed, and characterized to determine if the enzymes affinity for 1,3-PDO or NAD^+ plays a role in 3-HPA accumulation in *L. reuteri*.

Objectives of this study were:

- A) To clone and express the 1,3-PDO NAD^+ -dependent oxidoreductase from *L. reuteri*; and
- B) To determine the kinetic values for NAD^+ / NADH and 1,3-PDO for the oxidoreductase.

5.3 Materials and Methods

The expression vector pET-32b(+), *Escherichia coli* host Top10F' and the *E. coli* expression line BL21 were purchased from Merck, formally Novagen (San Diego, Ca, USA). Oligonucleotide primers were synthesized at Integrated DNA Technologies Incorporated (Toronto, ON, Canada). Genetic engineering enzymes (restriction enzymes, DNA ligase, DNA polymerase, etc.) were purchased from Fermentus, Invitrogen (Burlington, On, Canada), and New England Biolabs (Pickering, On, Canada). Plasmid DNA and PCR reactions were isolated and purified by the Bio Basic EZ-10 Spin Column Plasmid DNA miniprep (cat#. BS414) and EZ-10 Spin Column PCR products purification kit (cat# BS363) respectively (Markham, ON, Canada). Genomic DNA was isolated by the Wizard Genomic purification kit (A1120) from Promega (Madison, WI, USA). Other chemicals used in this research were ACS-grade or better and purchased from VWR international (Mississauga, ON, Canada).

5.3.1 Cloning of 1,3-propanediol NAD⁺ dependent oxidoreductase

The partially-annotated *Lactobacillus reuteri* DSM 20016 genome (NCBI Refseq: NC_009513) was used to identify a candidate 1,3-PDO NAD⁺-dependent oxidoreductase. The candidate gene, GeneID: 5188789, was the template for the cloning work. Using the candidate gene, GeneID: 5188789, primers were generated containing a EcoR V and Xho I restriction site on the 5' and 3' end, respectively, while removing the stop codon of the target gene. The 3' stop codon was removed to fuse the gene with a HIS tag on the carboxy terminal end of the recombinant protein. The primers used to amplify the 1,3-PDO NAD⁺-dependent oxidoreductase gene are provided in Table 5.1. Genomic DNA was isolated from a culture of *L. reuteri* DSM 20016 grown at 37°C in MRS by the Promega Wizard© genomic DNA purification kit following the manufacturer's suggested protocol. Platinum PfxDNA polymerase from Invitrogen was used to generate insert DNA through PCR with the primers. A temperature gradient PCR from 55°C to 65°C was utilized to define the optimal T_m and conditions for the generation of insert DNA. PCR conditions for the generation of insert DNA were as follows; 5 µl of 10x Buffer, 5 µl of 2 mM dNTP mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of template DNA, 0.5 µl of 50 mM MgSO₄, 5 units of Platinum Pfx DNA polymerase, and 14 µl of Milli-Q H₂O (ddH₂O).

The amplified DNA fragments from the successful PCR reaction were cleaned by the BioBasic EZ-10 Spin Column PCR Product Purification kit. An aliquot of the PCR reaction was run at 125 V for approximately 30 minutes in a 1% agarose gel to determine DNA concentration. An aliquot of isolated purified plasmid DNA was run at 125 V for approximately 30 minutes in a 1% agarose gel to determine plasmid DNA concentration. Insert and plasmid DNA were digested by EcoR V and Xho I. Digested samples were incubated in a water bath at 37°C for 18 – 24 hours. Following digestion the sample were purified by the BioBasic EZ-10 Spin Column PCR Product Purification kit and run on a 1% agarose gel to confirm purity and determine DNA concentrations. T4 DNA ligase from Fermentas was used to ligate insert and plasmid DNA. The ligation reactions contained molar ratios of insert to plasmid DNA of 1:3, 1:5, and 1:7, 2 µl of 10x Ligase Buffer, 2 µl of 50% PEG 4000, 7.5 units of T4 DNA ligase, and ddH₂O to a final volume of 20 µl. Ligation samples were incubated at 11°C for 18 – 20 hours. Following the incubation, the ligation mix was placed on ice for 5 minutes. After chilling the ligation mixture on ice for 5 minutes 1.5 µl was aliquoted to a 50-µl volume of freshly prepared Top10F' electrocompetent cells. The sample was electroporated in an Eppendorf 2510 electroporator (Mississauga, ON, Canada) using a 1-mm gap cuvette at 1400v. Immediately following electroporation, 1 mL of super optimal broth with catabolite repression (SOC) media was added to the cuvette and decanted to a sterile test tube. The tube was shaken at 75 rpm at 37°C for one hour post-electroporation. The sample was decanted to a 1.5-mL microtube and spun in a bench top centrifuge at 12,000 x *g* for 1 minute. The supernatant was removed and the cell pellet was resuspended in 100 µl of fresh SOC. The resuspended cell pellet was plated onto LB agar plates containing 150 µg/mL ampicillin and left to incubate at 37°C for 10 – 18 hours. Colonies were picked and grown in LB containing 150 µg/mL ampicillin. Plasmid DNA was isolated from cultures and PCR screening along with restriction digestion, and Sanger sequencing was utilized to identify positive clones.

Table 5.1 Primers utilized for cloning of the NAD⁺ dependent oxidoreductase

System	Orientation	Primer Sequence	Restriction Enzyme	Length	T _m	Cut Type
pET-32b(+)	Forward	5' CGGAGCTGAT <u>ATCAATGGAAAAAATATAGTATG</u> `3	EcoR V	32 bp	55.7	Blunt
pET-32b(+)	Reverse	5' ATAGTAC <u>TCGAGACGAATTATTGCTTCGTAA</u> `3	XhoI	31 bp	57.3	Sticky

*Underlined text denotes cut sites; Bolded text denotes sequence complementary to the gene of interest

Plasmid DNA from positive clones was isolated by the Biobasic EZ-10 Spin Column Plasmid DNA Miniprep kit. Isolated positive plasmid DNA was re-transformed into the *E. coli* BL21 cell line with the same method as described above, and plated on LB agar plates. Colonies were picked and grown in LB containing 150 µg/mL ampicillin. Plasmid DNA was isolated from cultures and restriction digestion was utilized to identify positive clones.

5.3.2 Expression of recombinant 1,3-PDO oxidoreductase

Positive clones in the BL21 cell line were grown in LB broth containing 150 µg/mL of ampicillin at 37°C and shaken at 150 – 200 rpm in 500-mL baffled flasks. Optical density (OD) of the cultures was monitored in a Beckman Coulter DU800 spectrophotometer at an OD₆₀₀. Cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) when an OD₆₀₀ between 0.4 and 0.6 was observed to a final concentration of 1 mM IPTG. Immediately following induction cultures remained at 37°C or were moved to 25°C, or 20°C incubators and left to grow for an additional 24 hours shaking at 225 rpm.

Following a 24 hour incubation period the cultures were spun at 12,000 x *g* in a GS3 rotor in a Sorval high-speed centrifuge at 4°C for 20 minutes. The supernatant was decanted and the cultures were resuspended in 10 mM Tris-HCl (pH 8.0) per 50 mL of culture. The resuspended cell mass was disrupted by a Branson Sonicator (Danbury, CT, USA) S-450A. Cells were pulsed continuously for 5 minutes at an intensity of 4 with a duty cycle of 10 % on ice. The sonicated cell mass was spun for 15 minutes at 11,200 x *g* in an GS3 rotor in a Sorval high speed centrifuge at 4°C and the supernatant was decanted. SDS-PAGE (10% gel) was utilized to determine protein content in both the cell pellet and supernatant. The supernatant was concentrated by an Amicon ultrafiltration cell using a 30 kDa filter. Protein concentration at all stages was determined by a Bradford assay.

5.3.3 Determination of recombinant oxidoreductase kinetics

Due to the inability to obtain or generate ACS grade 3-HPA as a substrate, 1,3-PDO was used as the substrate in the formation of 3-HPA. Kinetic values for the recombinant 1,3-propanediol NAD⁺-dependent oxidoreductase were determined following the protocol of

Johnson et al (Johnson and Lin 1987). Enzymatic activity was determined spectrophotometrically at 340 nm at 25°C by the formation of NADH. The assay contained 100 mM 1,3-PDO, 0.6 mM NAD⁺, 30 mM ammonium sulfate and 100 mM potassium bicarbonate buffer at a pH of 9.0 in a total final volume of 1-mL. Units of activity are determined in micromoles per minute at 25°C.

5.4 Results

Isolation of genomic DNA and temperature gradient PCR yielded significant quantities of insert DNA at a T_m of 55.3°C. Candidate colonies were digested and positive colonies were confirmed by restriction digestion with EcoR V and Xho I as seen in Figure 5.1.

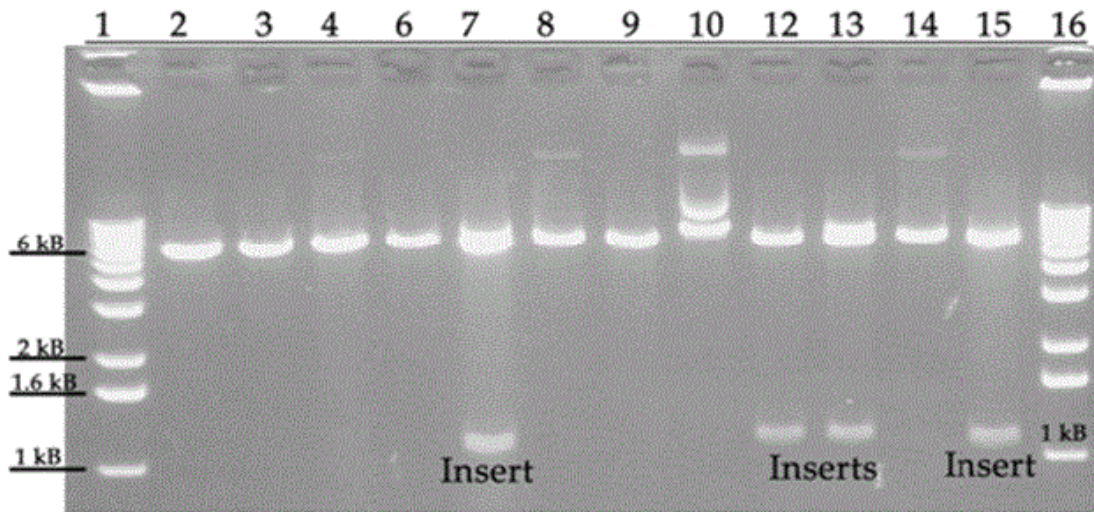


Figure 5.1 Restriction digestion of pET-32(b)+ candidate NAD⁺ dependent oxidoreductase clones

Candidate colonies were digested with EcoR V and Xho I at 37°C for 16 hours. Positive colonies displayed a band at 5.8 kb and 1.1 kb. Negative colonies display a band at 5.8 kb only. Positive colonies can be seen in lanes 7, 12, 13, and 15. A Fermentas GeneRuler 1 kb plus DNA ladder can be seen in lanes 1 and 16. Potential positive colonies can be seen in lanes, 7, 12, 13, and 15.

Candidate colony plasmid DNA was sent for Sanger sequencing. Sequence analysis confirmed positive clones in the TOP10F' cell line. They included a number of clones containing with HIS tags on both N and C terminal (#7, 12, 13) and a clone containing a HIS tag at the C terminus only (#15).

5.4.1 Expression of recombinant 1,3-PDO oxidoreductase

Following transformation and confirmation of positive colonies in the BL21 cell line the protein content of confirmed positive colonies grown and expressed at 37°C was observed by SDS-PAGE analysis as seen in Figure 5.2. Protein content of cultures grown at 37°C and expressed at 20°C can be seen in Figure 5.3.

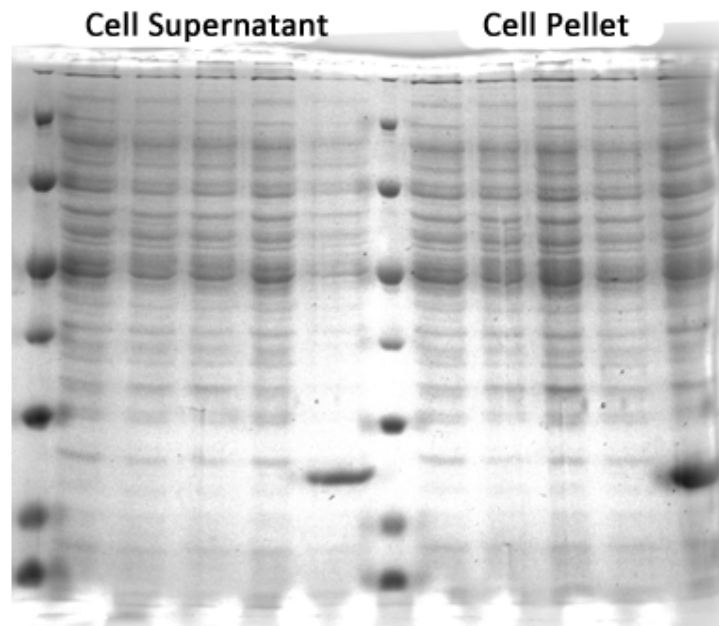


Figure 5.2 A 10% SDS-PAGE gel of candidate recombinant NAD⁺ dependent oxidoreductase clones grown and expressed at 37°C. Candidate NAD⁺ dependent oxidoreductase clones were grown to an OD₆₀₀ of 0.5. Cultures were induced with IPTG to a final concentration of 1 mM and expressed for 24 hours at 37°C in a baffled flask at 225 rpm. A sample was spun for each candidate and the supernatant and pellet were run analyzed by SDS-PAGE.

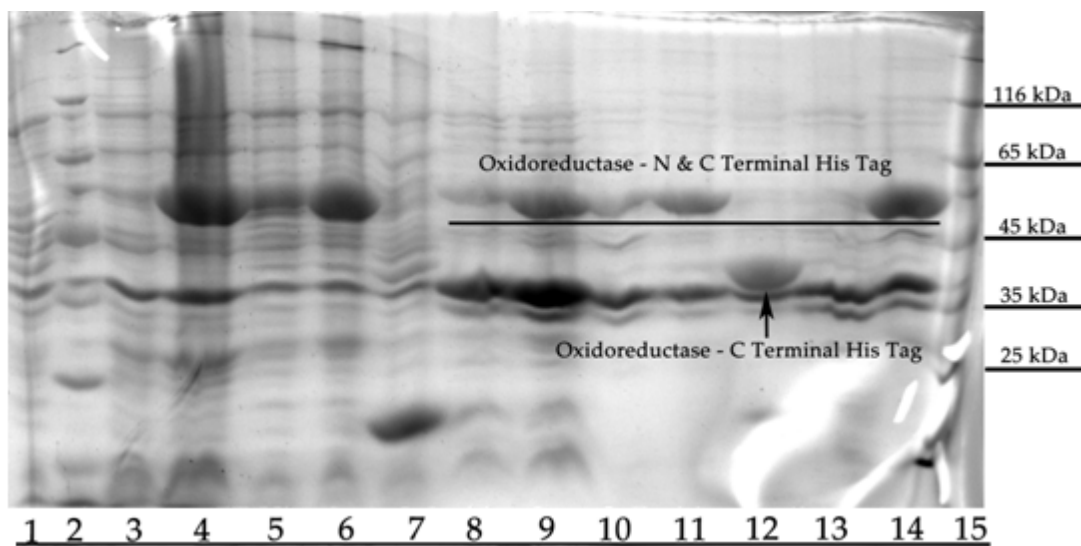


Figure 5.3 10% SDS-PAGE gel of candidate recombinant NAD⁺ dependent oxidoreductase clones grown and expressed at 20°C

Candidate NAD⁺ dependent oxidoreductase clones were cultured and induced with IPTG for 24 hours at 20°C. Soluble proteins (crude extracts) and insoluble proteins (cell debris) were examined on a 10 % SDS-PAGE; Lane 1, Empty pET-32(b)+; 2, molecular weight marker; 3-7, insoluble protein fractions of samples #1-5; 8-12, soluble protein fractions of samples #1-5; 13, insoluble protein fraction of sample #6; 14, soluble protein fraction of sample #6; 15, molecular weight marker.

SDS-PAGE analysis revealed two distinct protein bands between 65 and 45 kDa and 45 and 35 kDa. The dominant bands correlate with the theoretical size expected for the recombinant protein containing a single or double HIS tag. To determine if the recombinant proteins were expressed in an active form, an aliquot of the crude extract was assayed for activity.

It was determined that the larger dominant band containing both the N and C terminal HIS tag lacked activity (Figure 5-4). Conversely, the recombinant enzyme with only the C terminal tag demonstrated activity (Figure 5-4).

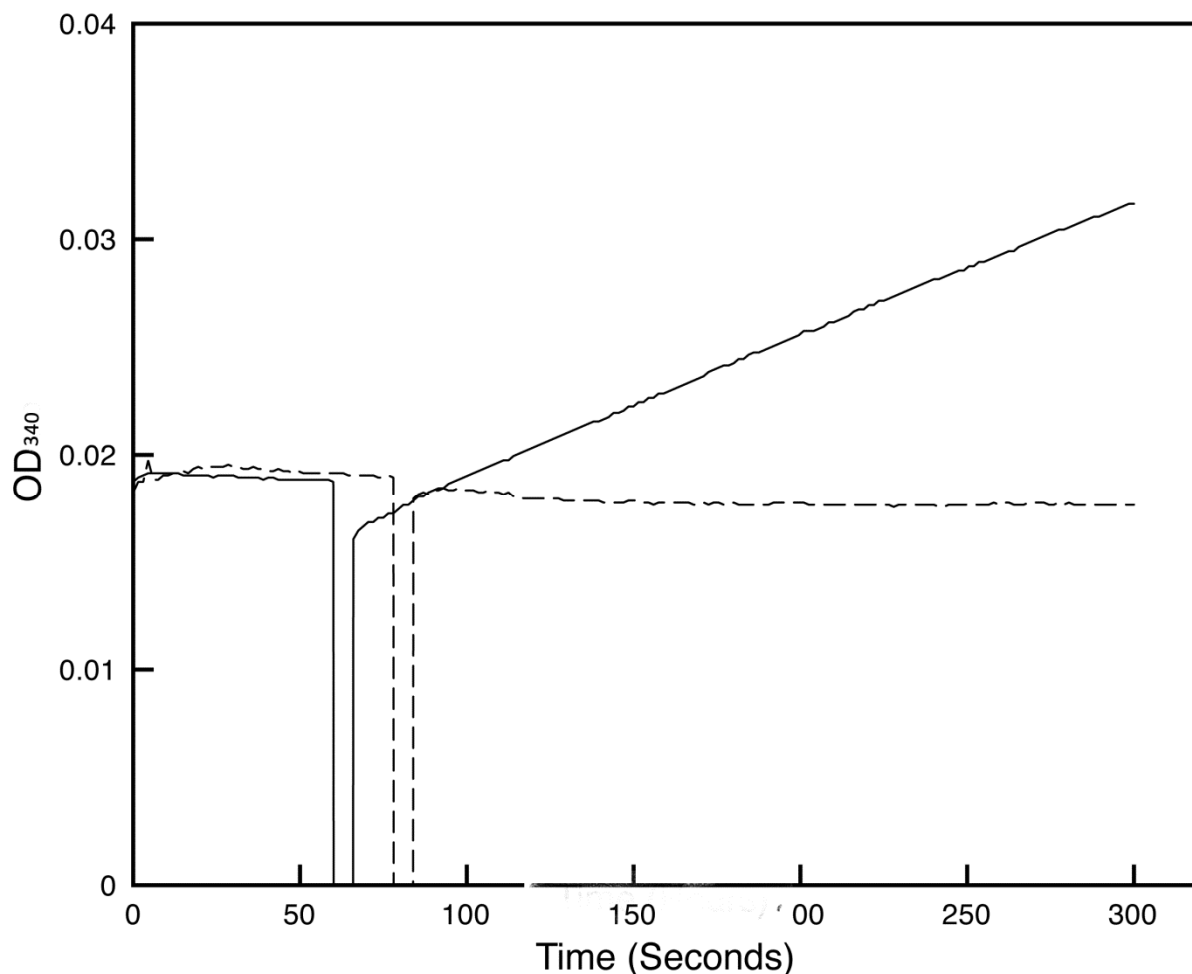


Figure 5.4 Activity of candidate NAD⁺ dependent oxidoreductase clones.

Enzymatic activity was determined spectrophotometrically at 340 nm at 25°C by the formation of NADH. The assay contained 100 mM 1,3-PDO, 0.6 mM NAD⁺, 30 mM ammonium sulfate, and 100 mM potassium bicarbonate buffer with a 15-μL aliquot of (— — —) N and C terminal Oxidoreductase tags, and (—) C terminal Oxidoreductase tag at a pH of 9.0 in a total volume of 1-mL

5.4.2 Determination of recombinant oxidoreductase kinetics

To determine the V_{\max} and k_{cat} of 1,3-PDO for NAD⁺-dependent oxidoreductase, the concentration of 1,3-PDO was varied from 5 mM to 50 mM. The assay was performed as per the protocol of Johnson and Lin (Johnson and Lin 1987). The activity was determined by rearranging Beers law to equation 5.1. Equation 5.1 defines the activity of the recombinant enzyme as the number of μmol per minute.

$$C = \frac{\left(\frac{ABS}{\varepsilon \cdot L} \right) \cdot 0.001mL}{\left(\frac{2}{60} \right)} \quad \text{(Equation 5.1)}$$

Using the calculated protein concentration determined by a Bradford assay as $27.5 \pm 0.2 \mu\text{g}/\mu\text{l}$. The V_{max} and K_m values were determined using the Lineweaver-Burke plots. The $V_{\text{max}}^{1,3-PDO}$ value was $0.65 \pm 0.07 \mu\text{mol}$ per minute per mg of crude extract. The $K_m^{1,3-PDO}$ value was $28.4 \pm 1.1 \text{ mM}$. The K_m and V_{max} values for NAD^+ for the recombinant enzyme in the crude extract were determined as previously described with two changes in the conditions of measurements. To determine the kinetic values associated with NAD^+/NADH the concentration of 1,3-PDO was maintained at 150 mM to ensure excess substrate. The second amendment was to vary the concentration of NAD^+ from 0.1 mM to 1.5 mM . The calculated $V_{\text{max}}^{\text{NAD}^+}$ value was $0.50 \pm 0.19 \mu\text{mol}$ per minute per mg of crude extract. The $K_m^{\text{NAD}^+}$ value was $1.28 \pm 0.57 \text{ mM}$. The final K_m and V_{max} values for both 1,3-PD and NAD^+ in the crude extract are as shown in Table 5.2.

Table 5.2 Kinetics of recombinant NAD+ oxidoreductase

Substrate	K_m (mM)	V_{max} ($\mu\text{mol}/\text{minute} \cdot \text{mg}$)
1,3-PDO	23.8 ± 1.1	0.56 ± 0.07
NAD^+	1.28 ± 0.57	0.50 ± 0.19

5.5 Discussion

The ability of *L. reuteri* to survive in, and accumulate large concentrations of 3-HPA, is of great interest given the detrimental effects of 3-HPA in other bacteria (Talarico and Dobrogosz 1989; Barbirato et al. 1996; Zheng et al. 2011). Further, the production of 1,3-PDO by *L. reuteri* has been shown to be dependent on the ratio of glucose to glycerol and the NAD^+/NADH pool (Luthi-Peng et al. 2002). Given the requirements for the production of 1,3-PDO in *L. reuteri*, determining the optimal conditions for activity of glycerol dehydratase and NAD^+ -dependent 1,3-PDO oxidoreductase may aid in increasing the final yield of 1,3-PDO in other 1,3-PDO producing bacteria (Luthi-Peng et al. 2002).

Industrial biotechnology has an additional interest in cloning the NAD^+ -dependent 1,3-PDO oxidoreductase. By cloning enzymes such as glycerol dehydratase and the NAD^+ -dependent 1,3-PDO oxidoreductase into optimized industrial recombinant systems the titer of 1,3-PDO may be greatly increased. Initial work, as seen in Appendix A, was able to produce clones containing glycerol dehydratase. Unfortunately, the glycerol dehydratase-containing clones were unable to express the recombinant protein in an active soluble form. Efforts to clone the NAD^+ -dependent 1,3-PDO oxidoreductase were undertaken, not only as a means of characterizing the enzyme, but to eventually explore the dynamic interaction of glycerol dehydratase and the NAD^+ -dependent 1,3-PDO oxidoreductase in producing and mitigating the deleterious effects of 3-HPA. Restriction digestion and sequence analysis confirmed a number of candidate colonies containing the proposed NAD^+ oxidoreductase from *L. reuteri* (GeneID: 5188789).

Following sequence confirmation the series of candidate colonies were tested for their ability to express the recombinant protein at various temperatures. Initial expression experiments yielded no recombinant protein. Work done by Li et al. indicated that lower temperatures produced an active soluble recombinant NAD^+ -dependent 1,3-PDO oxidoreductase (Li et al. 2011). Subsequent expression experiments determined the recombinant protein was successfully expressed as a soluble active protein at 20°C. Candidate recombinant oxidoreductase clones expressed at 20°C yielded two distinct protein bands. The pET-32b(+) expression system is capable of expressing proteins with N terminal HIS, Trx, and S-protein tags and a C terminal HIS tag. The expressed size of the two recombinant proteins

match the theoretical value for a recombinant protein containing an N and C terminal HIS tag as well as a single HIS tag, respectively. DNA sequence analysis confirmed the active candidate colonies contained a HIS tag in frame at the C terminus. Sequence analysis confirmed the loss of the N terminal HIS tag in the active recombinant protein. Both forms of the recombinant protein were assayed for activity. Results demonstrated that the recombinant protein containing both the N and C terminal HIS tag lacked activity (Figure 5.4). A lack of activity may be due to the N terminal tags sterically hindering the active site. The clone containing the single HIS tag demonstrated significant activity and was used to determine K_m and V_{max} for the recombinant protein.

The K_m value for 1,3-PDO was lower than expected indicating the enzyme has a lower affinity for 1,3-PDO than NAD^+ . The data may not be fully representative of the kinetics of the recombinant enzyme as purification and subsequent refined kinetic analysis has yet to be achieved. Although the data is sufficient to conclude the recombinant oxidoreductase binds NAD^+ with a far greater affinity than 1,3-PDO. The affinity for the cofactor $NAD^+/NADH$ gives further credence to the argument that the intracellular levels of $NAD^+/NADH$ play a vital role in the catalysis of 3-HPA to 1,3-PDO. As such, increased quantities of 1,3-PDO may be generated by altering the metabolism of the organism to generate or shuttle additional quantities of $NAD^+/NADH$ to the 1,3-PDO pathway (Horng et al. 2010). The principal of increased $NAD^+/NADH$ intracellular concentration may be in part demonstrated by the antithesis. An examination of the fed-batch trials of Study 2 demonstrated that additional quantities of glucose led to an increase in cell density not 1,3-PDO concentration. Additionally, Study 1 found that the titer of ethanol correlated with increasing concentrations of glucose from 5 – 50 mM; whereas the concentration of 1,3-PDO varied only slightly with increasing glucose concentrations. The previous two examples demonstrate that simply providing additional energy resources is insufficient to increase 1,3-PDO production. Rather, increasing the levels of $NAD^+/NADH$ specifically for the 1,3-PDO pathway are required to increase yields (Horng et al. 2010). As the metabolic profile can drastically affect the yield of 1,3-PDO the use of a closely related system with a known metabolic pathway such as *L. reuteri* may provide an optimal model system to gauge the effects of various conditions on the production of 1,3-PDO.

5.6 Conclusion

The high substrate affinity for NAD^+/NADH suggests that the availability of NAD^+/NADH may be a determining factor in 1,3-PDO titer. The generation of NAD^+/NADH is dependent upon the oxidative branch of metabolism and the production of 1,3-PDO occurs in fermentative metabolism. By taking into account the determined affinity for NAD^+/NADH , the production of NADH under oxidative metabolism and the recycling of NAD^+/NADH under fermentative metabolism the importance of limiting the competition for NAD^+/NADH becomes apparent. Understanding that the metabolic and redox-balance of the organism via NAD^+/NADH plays a more vital role in 1,3-PDO production suggests that *L. reuteri* may serve as an ideal model organism for determining the effects of various culture conditions on the production of 1,3-PDO.

6. GENERAL DISCUSSION

Data collected in Study 1 demonstrated that the optimized production of 1,3-PDO from *L. panis* is dependent upon a series of specific culture conditions. Specifically, it was determined that the concentration of glycerol, glucose, and pH play a vital role in determining the titer of 1,3-PDO produced. Further, previous results of our research group demonstrated the inability of the *L. panis* PM1 isolate to survive solely on glycerol (Khan et al. 2012). However, what is more important is understanding why these conditions lead to an increased production of 1,3-PDO. In order to understand why the various conditions lead to an increased production of 1,3-PDO data from Studies 1- 3 must be examined from a systems perspective.

The inability of *L. panis* to survive solely on glycerol indicates the failure of the organism to convert glycerol to DHAP or G3P, thereby limiting the pathways available to the organism to produce the necessary energy and reducing equivalents (e.g; ATP, FAD/FADH₂, NAD⁺/NADH/NADPH) from alternative sources.

As the organism lacks the capability to produce various reducing equivalents the importance of oxidative metabolism in generating a sufficient supply of reducing equivalents becomes heightened. This importance was demonstrated in Study 1 examining the effects of aeration and various concentrations and types of primary carbon sources. Study 1 demonstrated the negative impact of aeration and the importance of glucose in 1,3-PDO production. The importance of glucose exemplifies the significance of generating and maintaining the NAD⁺/NADH pool in the organism. The negative effects associated with aeration are thought to occur as in the presence of oxygen NADH oxidase would be used to recycle NADH not the NAD⁺-dependent oxidoreductase in 1,3-PDO production (Kang, Korber, and Tanaka, personal correspondence). Therefore one may believe that simply increasing the supply of reducing equivalents through a supply of energy sources will result in an increase in 1,3-PDO. Nevertheless, it was found that the effect of increasing glucose concentration on 1,3-PDO production was negligible. However, the positive correlation between ethanol concentration

and glucose concentration illustrates that intracellular competition for energy and reducing equivalents also plays a vital role in the final concentration of 1,3-PDO produced. The effects of competition were also seen in Study 2 when increasing glucose concentration led to an increase in cellular growth but not 1,3-PDO production. Finally, the kinetic values for the NAD^+ -dependent oxidoreductase in Study 3 illustrate the substrate affinity for NAD^+/NADH indicating its availability may be a determining factor in 1,3-PDO concentration and 3-HPA accumulation. Therefore, the conditions found for the optimal production of 1,3-PDO can be explained by understanding their effect on shifting the redox-balance of the organism. By shifting the redox-balance of the organism to require the recycling of NAD^+/NADH through the 1,3-PDO pathway, increased concentrations of product may be achieved.

7. GENERAL CONCLUSIONS

The production of G3P and or DHAP in organisms such as *K. pneumoniae* allows them to survive solely on glycerol (Streekstra et al. 1987). The inability of *L. panis* to survive solely on glycerol indicates the absence of pathways responsible for the generation of either G3P or DHAP from glycerol. Given the inability of the *L. panis* isolate to utilize glycerol as a primary carbon source, alternative carbon sources will be required for its survival. The inability of the *L. panis* PM1 isolate to produce either G3P or DHAP further indicates that under carbon limited or fermentative conditions it is likely glycerol is utilized solely as an alternative hydrogen acceptor as seen in *L. reuteri* (Talarico et al. 1990).

As demonstrated in Study 1 glucose has been shown to be the preferred primary carbon source for *L. panis* for both the accumulation of cell mass and the production of 1,3-PDO. Further, as demonstrated in Study 1, carbon limited conditions were required for the production of 1,3-PDO where a correlated increase in ethanol concentration was observed with an increasing concentrations of glucose. The increase in ethanol concentration associated with increasing glucose concentration indicates that under carbon limited and fermentative conditions, the pathway responsible for 1,3-PDO production is not a priority. It is hypothesized that the shift in priority leading to the generation of 1,3-PDO occurs due to a decrease in available NAD^+/NADH concentration. Data obtained in the pH trial in Study 1 indicated that culture conditions that put pressure on pathways linked to 1,3-PDO production, such as the formation of lactic and acetic acid, may allow for additional concentrations of 1,3-PDO to be produced. As the generation of NAD^+ and NADH is dependent upon the oxidative branch of metabolism and sufficient supply of a primary carbon source alternative mechanisms must be used to recycle NAD^+/NADH . The production of 1,3-PDO has been shown to be a mechanism to recover and recycle NADH and NAD^+ under fermentative and low carbon conditions in citrobacter and enterobacter (Johnson and Lin 1987; Talarico et al. 1990; Veigadacunha and Foster 1992). As a result during carbon limited or fermentative metabolism competition would exists for NAD^+/NADH among the

metabolic pathways responsible for producing energy products and maintaining the redox-balance of the system thereby limiting their available concentration to the 1,3-PDO pathway. Data obtained from the cloning and characterization of a native oxidoreductase from *L. reuteri* DSM20016 revealed high substrate affinity for NAD^+/NADH further suggesting that the availability of NAD^+/NADH may be a determining factor in 1,3-PDO titer. By taking into account the calculated affinity for NAD^+/NADH , the production of NADH under oxidative metabolism, and the recycling of NAD^+/NADH under fermentative metabolism the importance of limiting the competition for NAD^+/NADH becomes apparent. Further, maintaining an appropriate pool of NAD^+/NADH may ultimately limit the production of 1,3-PDO in our, and possibly other, microbial producers (Zheng et al. 2011).

Data obtained from the fed-batch fermentation of glycerol indicate that the sustained production of 1,3-PDO can be achieved by maintaining carbon limited conditions. Final concentrations of 1,3-PDO were found to be similar to reported levels for the *L. panis* isolate grown in static cultures containing glucose in the range of 5 – 50 mM producing 85.3 ± 11.9 versus 79.7 ± 10.4 mM 1,3-PDO for the fed-batch trial; however the duration and mole concentration of 1,3-PDO produced were significantly higher with 22.3 ± 1.6 versus 5.3 ± 0.7 mmol 1,3-PDO produced for the fed batch versus a static culture. The consistent molar concentration seen in both the static and fed-batch cultures may indicate the presence of an inhibitory product produced during 1,3-PDO production. Published data indicates that the intermediate 3-HPA plays a role as an inhibitor in 1,3-PDO production (Zheng et al. 2011). Given the required presence of 3-HPA in 1,3-PDO production it may be possible that a particular accumulation of 3-HPA inhibits 1,3-PDO production. Taking into consideration the accumulated data obtained in accordance with previously published results indicates that the optimized production of 1,3-PDO from the *L. panis* PM1 isolate will occur under conditions that provide the highest levels of NAD^+/NADH to the 1,3-PDO pathway while mitigating the negative effects of 3-HPA.

The defined optimized results lead to an increase in the concentration of 1,3-PDO obtained from the novel isolate. The increase in production, however, is insufficient at this time to warrant industrial interest for our isolate. Further experimentation regarding continuous and

fed-batch cultures mitigating the effects of toxic intermediates must be explored to appropriately determine the industrial relevance of our novel isolate.

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9. APPENDIX A – CLONING OF THE A AND B SUBUNITS OF GLYCEROL DEHYDRATASE FROM *L. REUTERI* DSM20016

The original objective was to determine and develop the capabilities of a recombinant glycerol dehydratase from *Lactobacillus reuteri* to form 3-HPA from waste glycerol. The recombinant clones are based upon the α and β subunits of the glycerol dehydratase operon from *L. reuteri*. The α subunit corresponds to the large subunit of the glycerol dehydratase responsible for the conversion of glycerol to 3-HPA (Talarico and Dobrogosz 1990; Barbirato et al. 1996; Toraya 2000). The β subunit corresponds to the reactivating factor associated with the glycerol dehydratase enzyme (Toraya 2000). The reactivating factor is thought to be responsible for replacing damaged and/or used coenzyme in the active α subunit (Mori and Toraya 1999; Tobimatsu et al. 1999; Toraya 2000; Kajiura et al. 2007).

The recombinant expression systems pKK-223 and pAL-781 were initially chosen as both systems are no longer under patent protection and for their ability to generate large amounts of recombinant protein. Clones of the α and β subunit were successfully generated in both the pKK-223 and pAL-781 systems. It was determined by restriction analysis and DNA sequencing that the pKK-223 and pAL-781 systems did contain the appropriate insert. However, conditions capable of producing an active soluble recombinant protein were unable to be determined. A closer review of the proteins expression indicated that the expression systems produced the protein as an insoluble inclusion body (Figures A.1, A.2, and A.3).

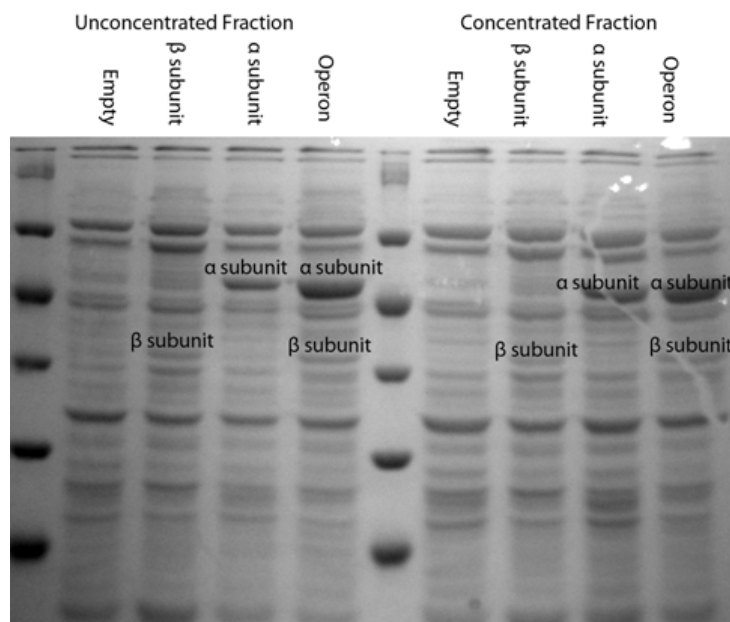


Figure A.1 Expression of recombinant Glycerol Dehydratase in pKK223-3. Expressed protein was concentrated in a 30 kDa filter and dialyzed overnight. A sample of the 70% soluble and insoluble fraction were run on a 10% SDS-PAGE gel.

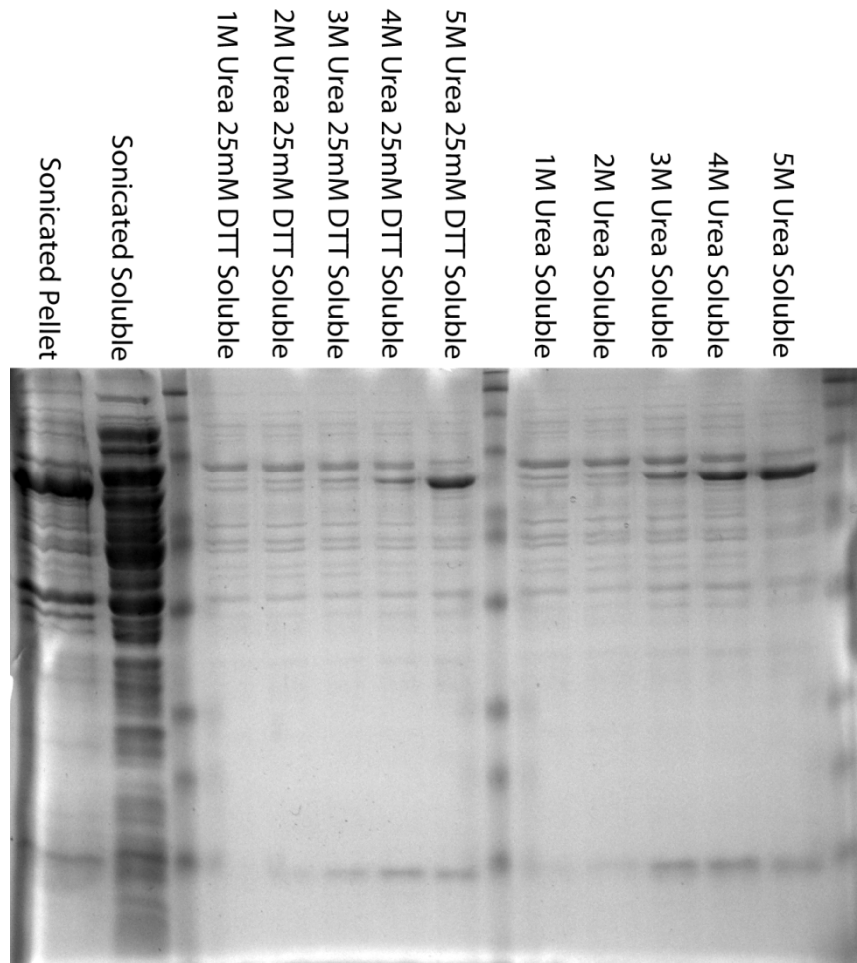


Figure A.2 Determination and solubilization of inclusion bodies expressed by recombinant glycerol dehydratase in pKK223-3. Recombinant glycerol dehydratase was pelleted and re-suspended in increasing concentrations of DTT and urea. The soluble fraction was pelleted and re-suspended until a final concentration of 5M urea was reached.

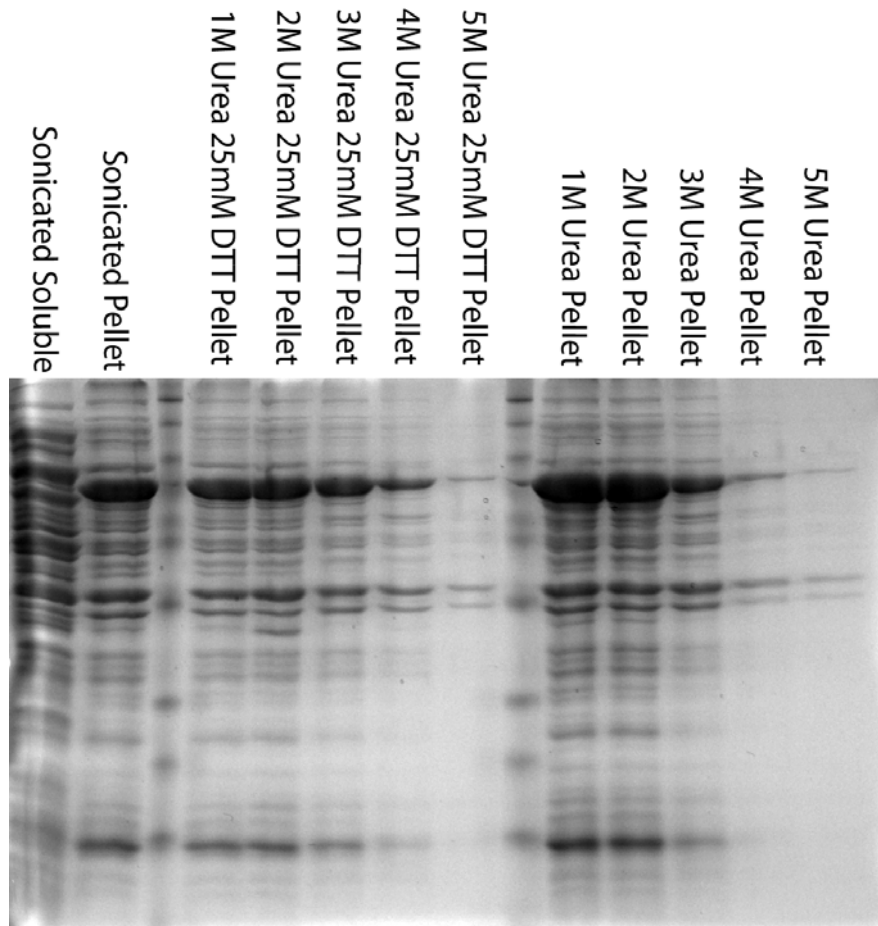


Figure A.3 Determination and solubilization of inclusion bodies expressed by recombinant glycerol dehydratase in pKK223-3.

Recombinant glycerol dehydratase was pelleted and re-suspended in increasing concentrations of DTT and urea. The insoluble fraction was pelleted and re-suspended until a final concentration of 5M urea was reached.

Mass spectrometry analysis confirmed the inclusion body produced in the pKK-223 α subunit clone was indeed the large subunit of glycerol dehydratase from *Lactobacillus reuteri* (Figures A.4 and A.5).

Mascot Search Results

```

User       : NRC Mascot Daemon
Email      : steve.ambrose@nrc-cnrc.gc.ca
Search title : PLGS processed pkl files searched on MASCOT, Ottawa
MS data file : \\DATA\MSPCDATA\MassSpec\Laboratory\Instruments\Q-ToF\PKL_files-2010\20100823_LR2_DTT.pkl
Database    : NCBIInr 28072010 (11505486 sequences; 3925745078 residues)
Taxonomy     : Bacteria (Eubacteria) (6499769 sequences)
Timestamp    : 24 Aug 2010 at 15:41:14 GMT
Protein hits : gi|148544953 glycerol dehydratase [Lactobacillus reuteri DSM 20016]
               gi|227545354 glycerol dehydratase [Lactobacillus reuteri CF48-3A]
               gi|2624772 Chain A, Crystal Structure Of The Asymmetric Chaperonin Complex GroELGROES(ADP)7
               gi|6225121 RecName: Full=60 kDa chaperonin; AltName: Full=Protein Cpn60; AltName: Full=groEL protein
               gi|6225122 RecName: Full=60 kDa chaperonin; AltName: Full=Protein Cpn60; AltName: Full=groEL protein
               gi|270290878 glycerol dehydratase large subunit [Pediococcus acidilactici 7_4]
               gi|15804600 malate synthase [Escherichia coli O157:H7 EDL933]
               gi|15802833 bifunctional NADH:ubiquinone oxidoreductase subunit C/D [Escherichia coli O157:H7 EDL933]
               gi|438187 heat-shock protein [Neisseria gonorrhoeae]
               gi|41081 unnamed protein product [Escherichia coli]
               gi|91791787 chaperonin GroEL [Shewanella denitrificans OS217]
               gi|238773816 GroEL [Candidatus Riesia pediculicola]
               gi|183675214 Cpn60 [uncultured soil bacterium]
               gi|148360427 penicillin binding protein 1A [Legionella pneumophila str. Corby]

```

Figure A.4 Mass spectrometry result of the dominant protein band in the 5M urea soluble fraction

Mascot Search Results

```

User       : NRC Mascot Daemon
Email      : steve.ambrose@nrc-cnrc.gc.ca
Search title : PLGS processed pkl files searched on MASCOT, Ottawa
MS data file : \\DATA\MSPCDATA\MassSpec\Laboratory\Instruments\Q-ToF\PKL_files-2010\20100823_LR1_urea_02.pkl
Database    : NCBIInr 28072010 (11505486 sequences; 3925745078 residues)
Taxonomy     : Bacteria (Eubacteria) (6499769 sequences)
Timestamp    : 24 Aug 2010 at 15:41:57 GMT
Protein hits : gi|148544953 glycerol dehydratase [Lactobacillus reuteri DSM 20016]
               gi|227545354 glycerol dehydratase [Lactobacillus reuteri CF48-3A]
               gi|42144 unnamed protein product [Escherichia coli]
               gi|1421648 Chain A, Conformational Variability In The Refined Structure Of The Chaperonin GroEL At 2.8 Angstrom Resolut
               gi|6225121 RecName: Full=60 kDa chaperonin; AltName: Full=Protein Cpn60; AltName: Full=groEL protein
               gi|291616009 NusA [Pantoea ananatis LMG 20103]
               gi|270290878 glycerol dehydratase large subunit [Pediococcus acidilactici 7_4]
               gi|15802949 phosphoenolpyruvate-protein phosphotransferase [Escherichia coli O157:H7 EDL933]
               gi|152972093 transcription elongation factor NusA [Klebsiella pneumoniae subsp. pneumoniae MGH 78578]
               gi|48783 ProRS [Escherichia coli]

```

Figure A.5 Mass spectrometry result of the dominant protein band in the 1M urea pellet fraction

The expression conditions for the pKK-223 α subunit clone were varied in an attempt to generate active soluble protein. Variations in the time, temperature, IPTG concentration, NaCl concentration and the addition of betaine and sorbitol were explored for both the growth and induction phases of the recombinant protein. No activity or soluble protein was detected. Alternative expression systems were explored and the *Pichia pastoris* expression system pHIL-S1 and the *E. coli* expression system pET-32b(+) were chosen as replacements to the pKK-223 and pAL-781 systems.

The pHIL-S1 system was chosen due to its ability to secrete recombinant protein into the media aiding significantly in protein purification. Further, the eukaryotic nature of the pHIL-S1 system was proposed as a means of combating issues of protein solubility and possible misfolding. The pET-32b(+) system was chosen due to its ability to add both a thioredoxin and a HIS tag to the recombinant protein. The thioredoxin and HIS tag were expected to aid in the solubility and purification of the recombinant protein respectively. Clones of the α and β subunits of glycerol dehydratase from *L. reuteri* were cloned into the pHIL-S1 and pET-32b(+) system. The complete operon of *L. reuteri* was also cloned into the pET-32b(+) system.

Initial SDS-Page, kinetic and NMR results of the pHIL-S1 containing clones showed low levels of expression and no detectable presence of 3-hydroxypropionaldehyde production. The predominate band seen in the expression for both pHIL-S1 clones was larger than expected (Figure A.6). Glycosylation of both the α and β subunit was proposed as a hypothesis to explain the increase in protein size. De-glycosylation of the protein samples was performed using an enzymatic means of cleavage under non-denaturing conditions.

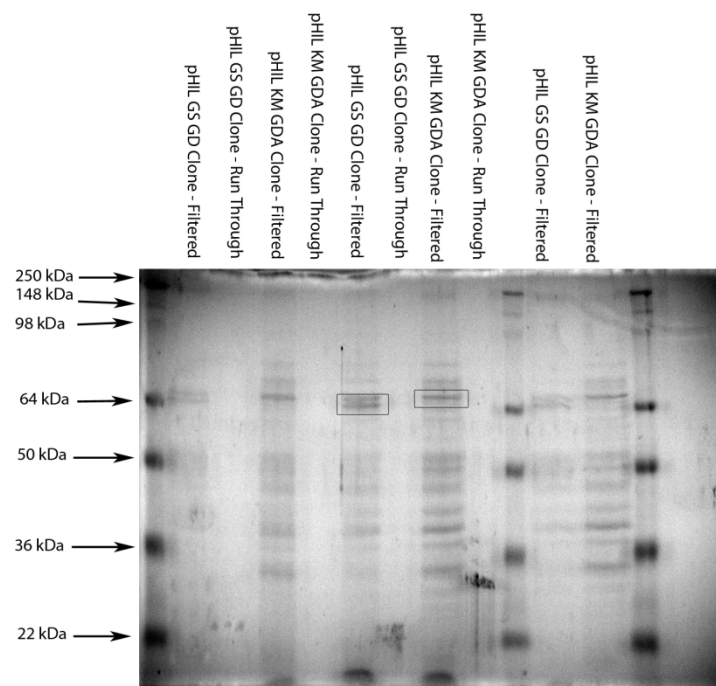


Figure A.6 Expression of alpha (GD) and beta (GDA) subunits of glycerol dehydratase in the pHIL-S1 clone. Boxes outline dominant band thought to be recombinant protein.

No detectable change in protein size was observed. Initial purification of the three pET-32b(+) clones indicate that large concentrations of recombinant protein are contained within the cell lysate (Figure A.7). Initial purification of the recombinant protein were discouraging as the expressed protein of the α subunit of glycerol dehydratase appears to be insoluble. Initial purification results for the complete operon were unable to produce a protein corresponding to the molecular weight of the α subunit. Further efforts must be taken place using other expression system, such as *Streptomyces* systems in the future.

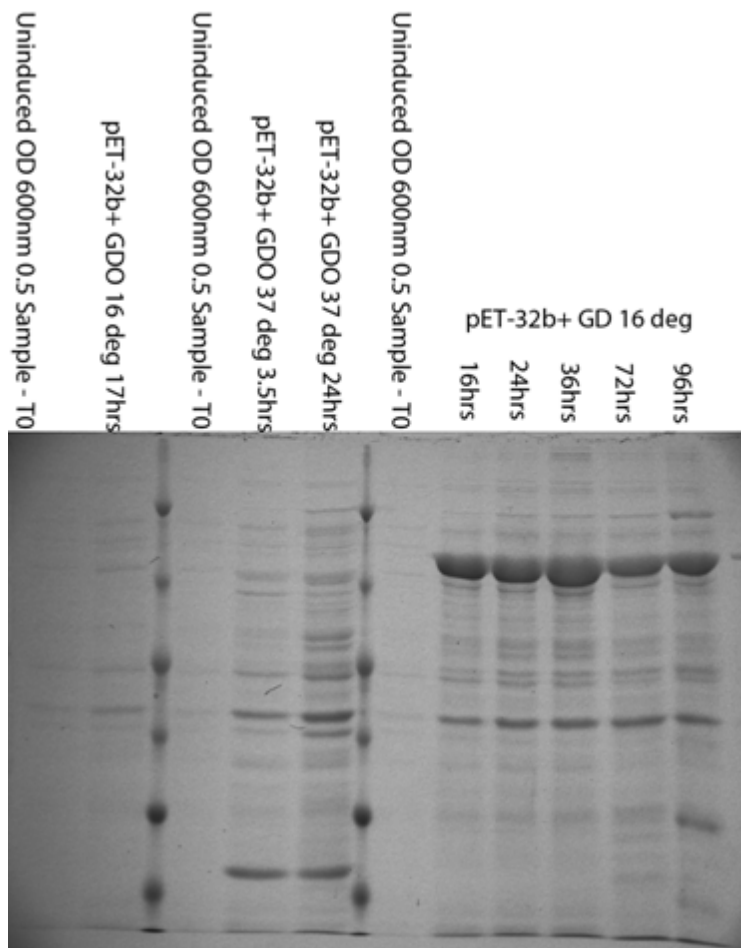


Figure A.7 pET-32b(+) expression of alpha subunit (GD) and glycerol dehydratase operon (GDO)

10. APPENDIX B – NMR SPECTRA OF *L. PANIS* PM1

Various NMR spectra were obtained during the course of Studies 1 - 3. The following NMR spectra demonstrate the ability of NMR to both identify and quantify the production of various metabolites (Ratanapariyanuch et al. 2011).

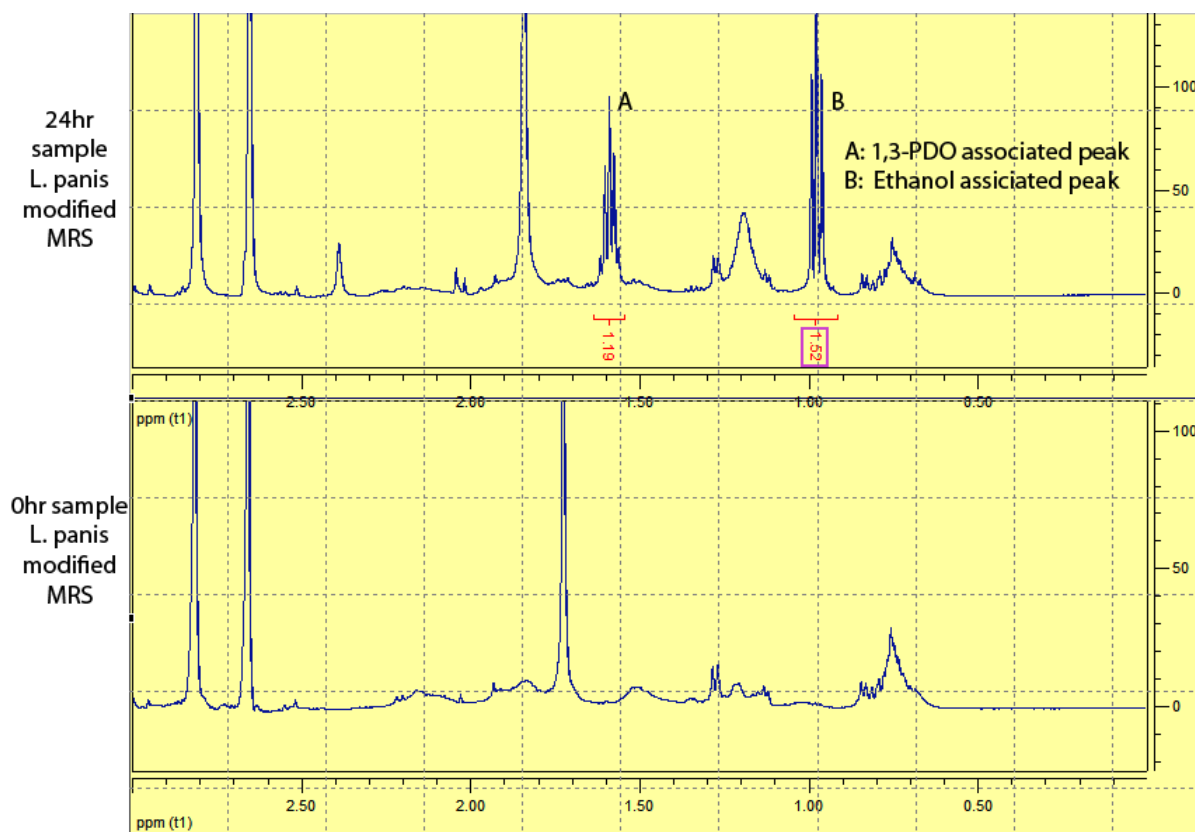


Figure B.1 NMR Spectra of *L. panis* grown in MRS and resuspended in mMRS at 0 and 24 hours.

Production of 1,3-PDO and ethanol were clearly shown as their corresponding peaks as indicated in the spectra

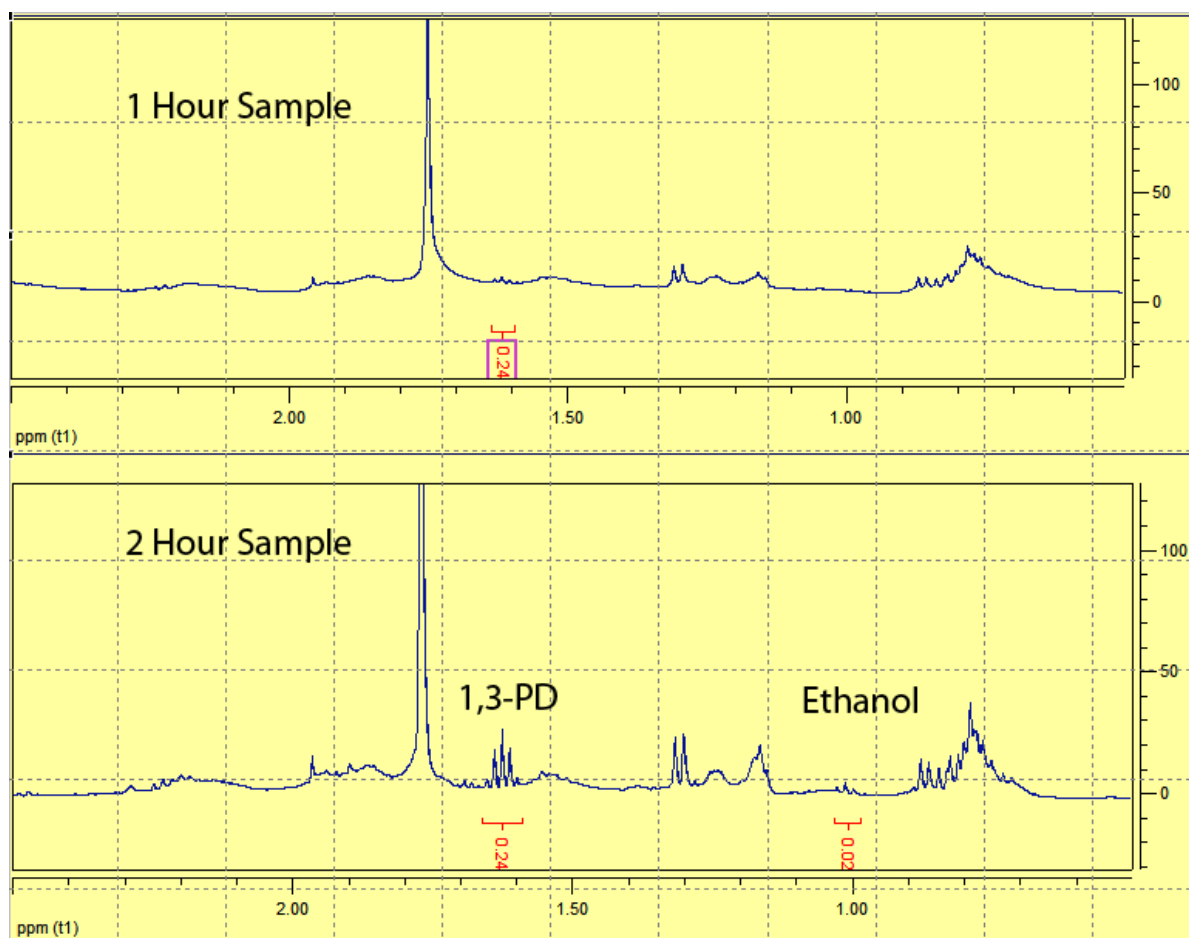


Figure B.2 NMR Spectra of *L. panis* grown in MRS and resuspended in mMRS at 1 and 2 hours.

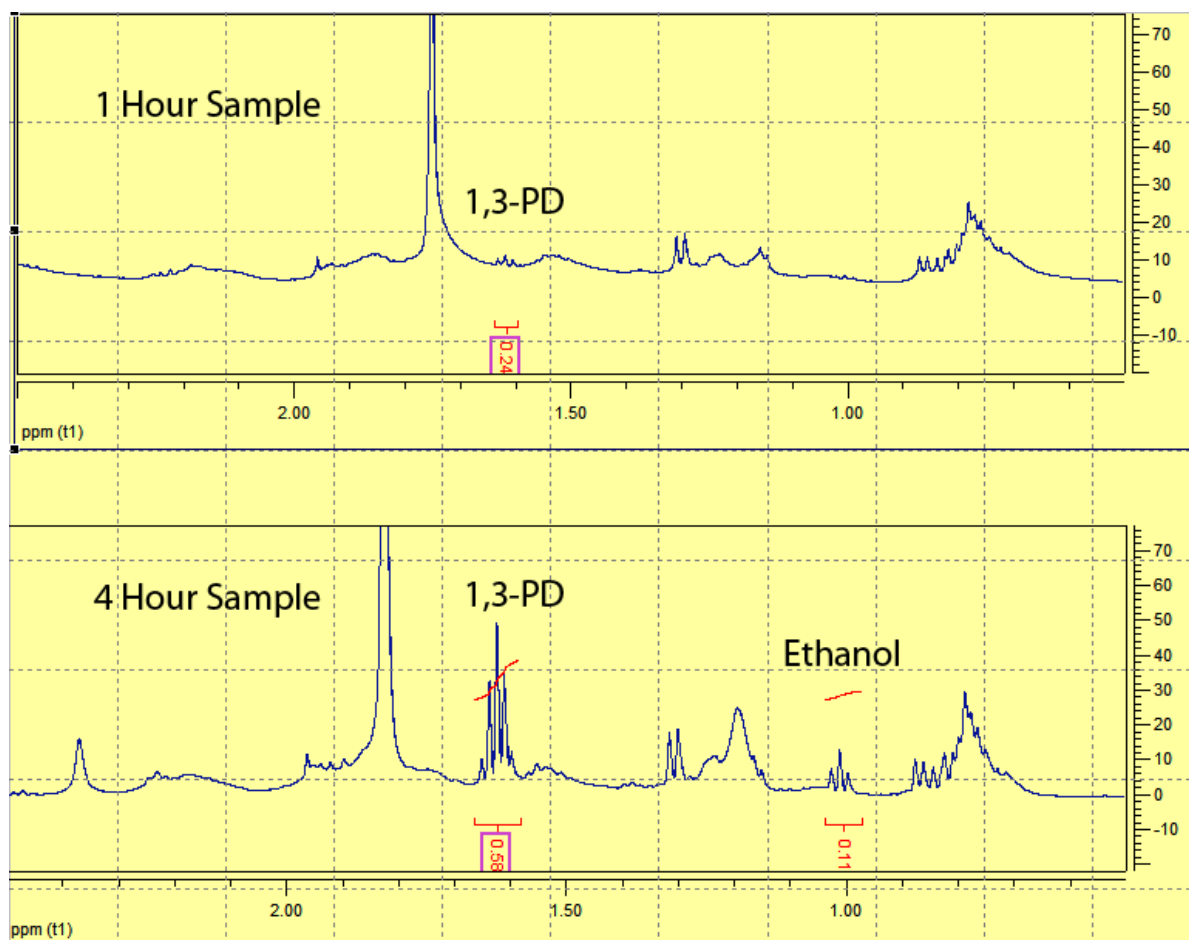


Figure B.3 NMR Spectra of *L. panis* grown in MRS and resuspended in mMRS at 1 and 4 hours

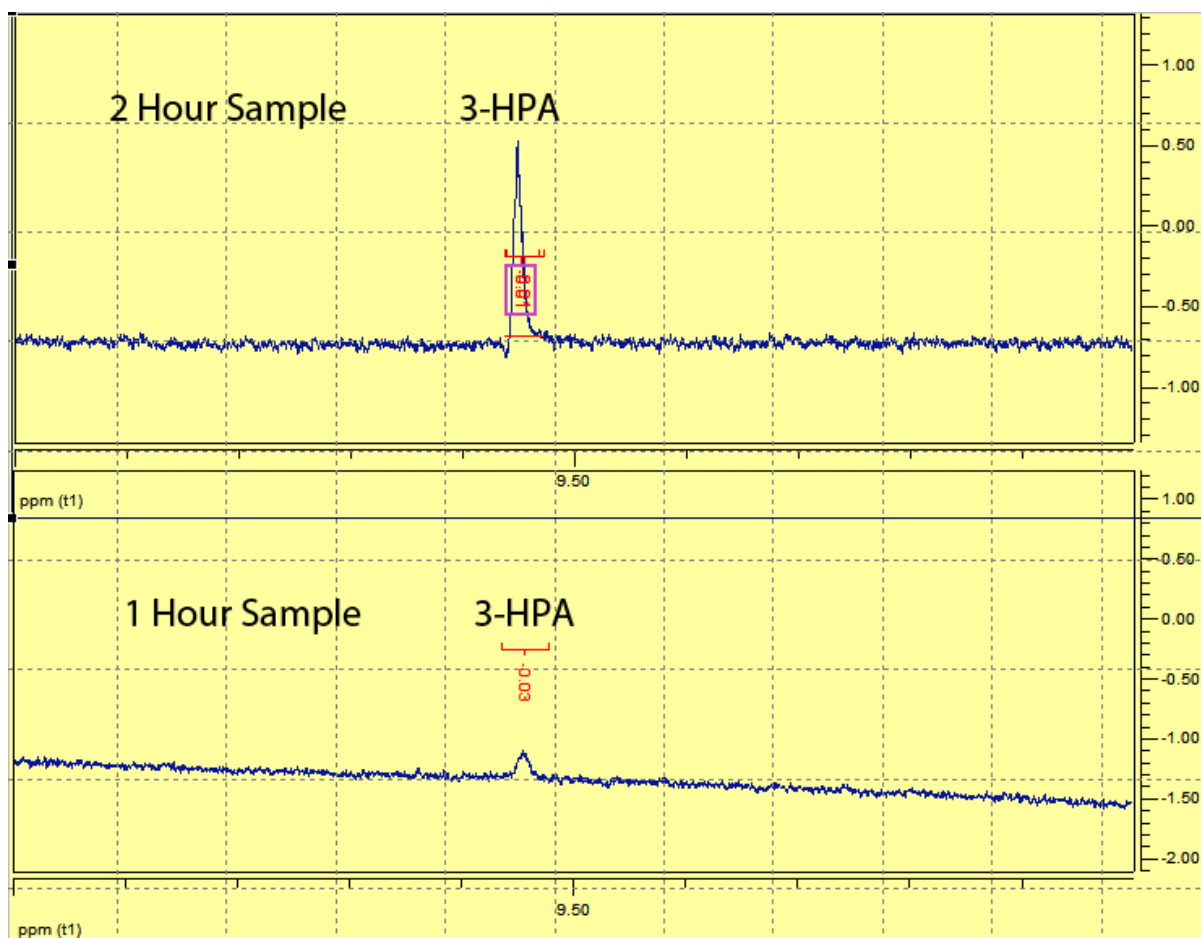


Figure B.4 NMR Spectra of *L. panis* grown in MRS and resuspended in mMRS at 1 and 2 hours

3-HPA was observed at 1 and 2 hours, although the height of the peaks were very small and the amount of the production is nearly zero compared to those of 1,3-PDO. This indicated 3-HPA was not accumulated during the culture of *L. panis* PM1 and it was immediately processed into 1,3-PDO.

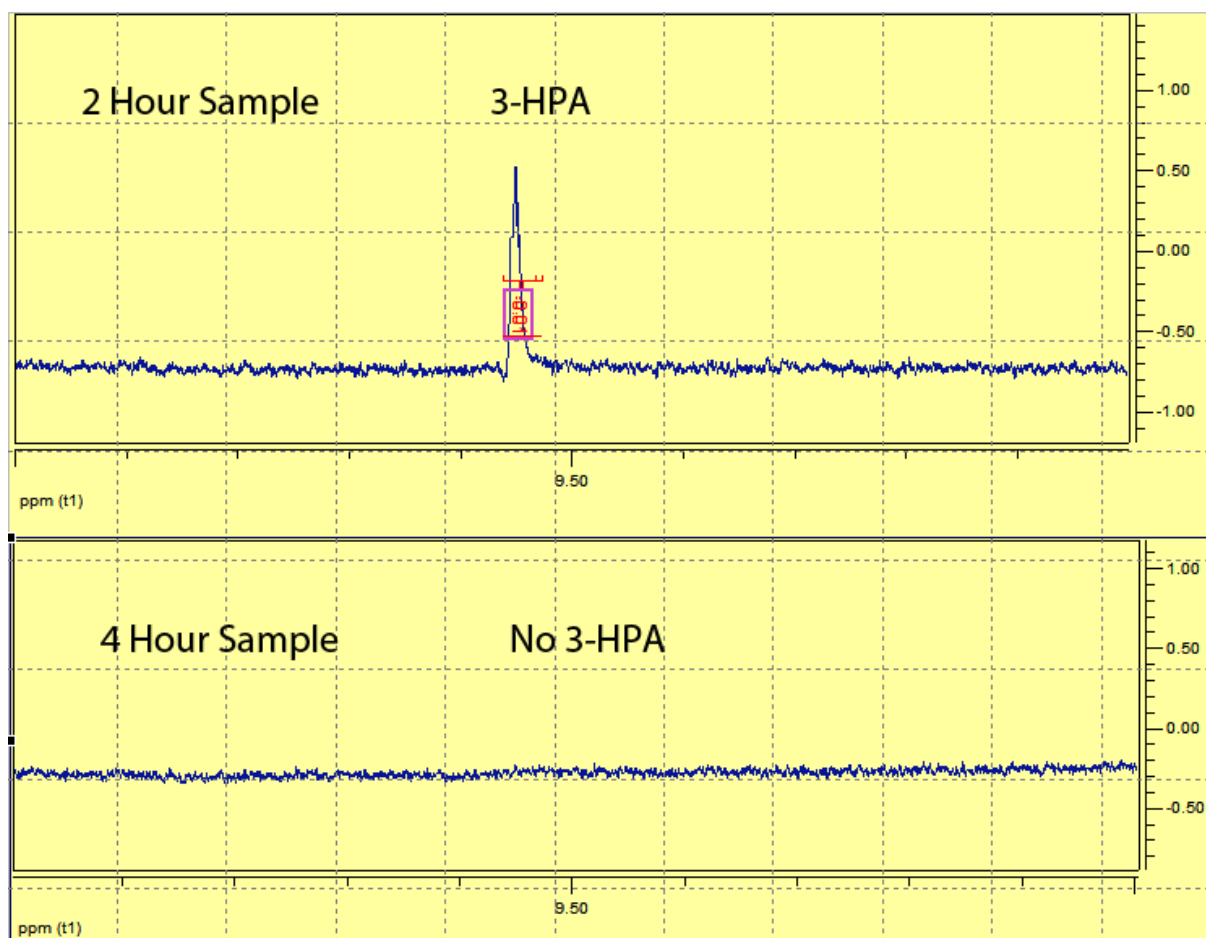


Figure B.5 NMR Spectra of *L. panis* grown in MRS and resuspended in mMRS at 2 and 4 hours

At four hours, 3-HPA was not observed, and it evidenced the rapid processing of 3-HPA into 1,3-PDO at the low glucose availability.